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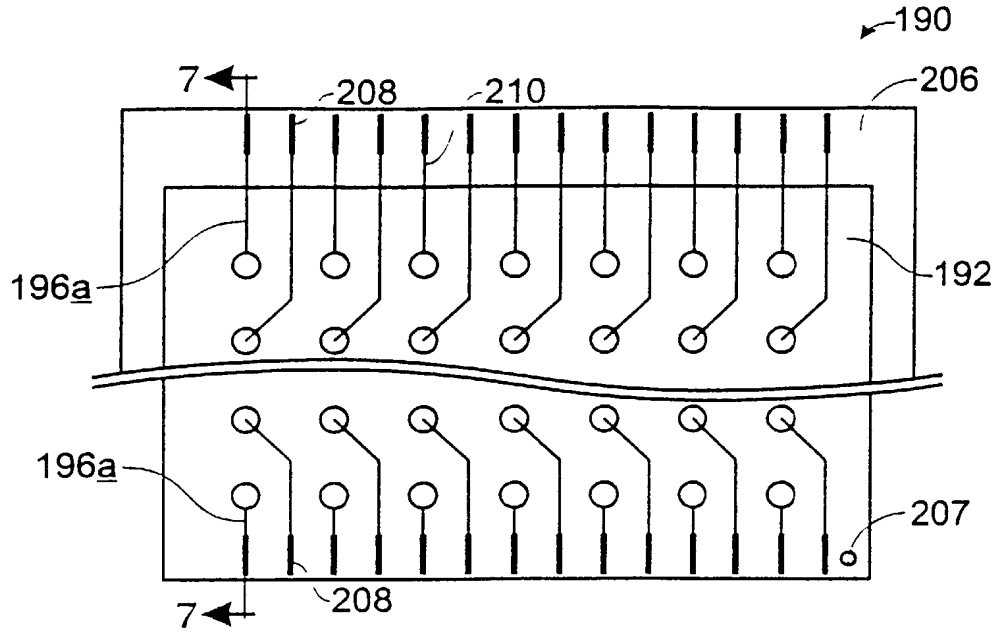
(54) Title: SAMPLE POSITIONING AND ANALYSIS SYSTEM



(57) Abstract: Systems for positioning and/or analyzing samples such as cells, vesicles, cellular organelles, and fragments, derivatives, and mixtures thereof, for electrical and/or optical analysis, especially relating to the presence and/or activity of ion channels.

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SAMPLE POSITIONING AND ANALYSIS SYSTEM

Cross-References

This application is based upon and claims the benefit of the following U.S. provisional patent applications, which are incorporated herein by reference: Serial No. 60/233,800, filed September 19, 2000, titled DESIGN OF HIGHLY INTEGRATED PHARMACEUTICAL SCREENING CHIPS, and naming Christian Schmidt as inventor; and Serial No. _____, filed September 13, 2001, titled HIGH-THROUGHPUT PATCH CLAMP SYSTEM, and naming Christian Schmidt as inventor.

This application incorporates by reference in their entirety for all purposes the following U.S. Patents: No. 5,355,215, issued October 11, 1994; and No. 6,097,025, issued August 1, 2000.

This application incorporates by reference in their entirety for all purposes the following patent applications: U.S. Patent Application Serial No. 09/581,837, filed July 28, 1998; U.S. Provisional Patent Application Serial No. 60/232,365, filed September 14, 2000; U.S. Provisional Patent Application Serial No. 60/233,800, filed September 19, 2000; U.S. Patent Application Serial No. 90/708,905, filed November 8, 2000; PCT Patent Application Serial No. PCT/IB00/00095, filed January 26, 2001; and PCT Patent Application Serial No. PCT/IB00/00097, filed January 26, 2001.

This application incorporates by reference in their entirety for all purposes the following U.S. Patent Applications: Serial No. 09/337,623, filed June 21, 1999; Serial

No. 09/349,733, filed July 8, 1999; Serial No. 09/478,819, filed January 5, 2000; Serial No. 09/596,444, filed June 19, 2000; Serial No. 09/710,061, filed November 10, 2000; Serial No. 09/722,247, filed November 24, 2000; Serial No. 09/759,711, filed January 12, 2001; Serial No. 09/765,869, filed January 19, 2001; Serial No. 09/765,874, filed January 19, 2001; Serial No. 09/766,131, filed January 19, 2001; Serial No. 09/767,434, filed January 22, 2001; Serial No. 09/767,579, filed January 22, 2001; Serial No. 09/767,583, filed January 22, 2001; Serial No. 09/768,661, filed January 23, 2001; Serial No. 09/768,765, filed January 23, 2001; Serial No. 09/770,720, filed January 25, 2001; Serial No. 09/770,724, filed January 25, 2001; Serial No. 09/777,343, filed February 5, 2001; Serial No. 09/813,107, filed March 19, 2001; Serial No. 09/815,932, filed March 23, 2001; and Serial No. 09/836,575, filed April 16, 2001; and Serial No. _____, filed August 20, 2001, titled APPARATUS AND METHODS FOR THE GENERATION OF ELECTRIC FIELDS WITHIN MICROPLATES, and naming James M. Hamilton as inventor.

This application incorporates by reference in their entirety for all purposes the following U.S. Provisional Patent Applications: Serial No. 60/223,642, filed August 8, 2000; Serial No. 60/244,012, filed October 27, 2000; Serial No. 60/267,639, filed February 10, 2001; Serial No. 60/287,697, filed April 30, 2001; Serial No. _____, filed August 2, 2001, titled pH PROBES FOR CELL-BASED FLUORESCENCE ASSAYS, and naming Zhenjun Diwu, Jesse J. Twu, Guoliang Yi, Luke D. Lavis, and Yen-Wen Chen as inventors; and Serial No. _____, filed August 31, 2001, titled KINETIC ASSAY

FOR DETERMINING CALCEIN RETENTION IN CELLS, and naming Kelly J. Cassutt, Jesse J. Twu, and Anne T. Ferguson as inventors.

This application incorporates by reference in its entirety for all purposes the following publications: Richard P. Haugland, Handbook of Fluorescent Probes and Research Chemicals (6th ed. 1996); and Joseph R. Lakowicz, PRINCIPLES OF FLUORESCENCE SPECTROSCOPY (2nd Ed. 1999).

Field of the Invention

The invention relates to systems for positioning and/or analyzing samples. More particularly, the invention relates to systems for positioning and/or analyzing samples such as cells, vesicles, cellular organelles, and fragments, derivatives, and mixtures thereof, for electrical and/or optical analysis, especially relating to the presence and/or activity of ion channels.

Background of the Invention

A variety of important biological processes occur at or within cell membranes. It therefore is not surprising that the biological function of membrane proteins has become an area of active research. Signal transduction processes in general, including nerve conduction, and neuroreceptors in particular have been shown to be influenced by pharmacologically active ingredients, making them obvious targets for drug development.[i] Ion channels and ion transporters also have been shown to be an important class of therapeutic targets. In fact, interactions with ion channels have become a major potential source of adverse effects when administering a therapeutic agent, leading the

Food and Drug Administration (FDA) and other government regulatory agencies to require safety profiling of potential therapeutics against certain ion channels.

This understanding of the interactions between potential drugs and cell membrane components is beginning to play a crucial role in modern drug development. In view of the increasing number of known receptors and the rapidly growing libraries of potential pharmaceutical ingredients, there clearly is a need for highly sensitive screening methods that permit the analysis of a large number of different substances with high assay throughput per unit time, otherwise known as "high throughput screening" (or "HTS"). In particular, there is a need for automated and/or high throughput screening methods that are relevant to cell membrane components.

At present, relatively traditional methods are used for the screening of pharmaceutical ingredients. Such methods include ligand binding assays and receptor function tests that are performed separately.[ii] Although binding assays are relatively inexpensive, and amenable to high throughput, they require labeled high-affinity ligands, and generally are limited to assays for ligands that can compete effectively for labeled ligand. Fluorescent or fluorogenic reagents generally are compatible with high throughput assays, including the analysis of ion channels using fluorescent calcium indicators, and the evaluation of membrane potential effects with potential-sensitive dyes. However, such reagents typically are not sensitive enough for single cell measurements, and generally can provide only indirect measurements of the membrane component of interest.

The patch clamp was introduced by Neher and Sakmann in the early 1980s as a powerful technique for the direct study of drug effects on single receptors. In recognition of the strength of the method, Neher and Sakmann were awarded the Nobel prize in 1991. Classical patch-clamp methods often are used in conjunction with functional membrane receptor assays, including receptors coupled to G-proteins and ion channel-forming receptors.[iii] This method is highly specific and extremely sensitive: it can, in principle, be used to measure the channel activity of individual receptor molecules. In doing so, glass micropipettes with an opening diameter of typically 1–0.1 μm are pressed on the surface of a biological cell. The membrane surface that is covered by the micropipette is called a "patch." If the contact between the glass electrode and the cell membrane surface is sufficiently electrically isolating, the ion flow over the membrane patch can be measured electrically with the aid of microelectrodes, one placed in the glass pipette and the other placed in the milieu opposite the membrane.[iv] A key advantage of this electrophysiological method is that it makes directly accessible the function of the corresponding channel-forming proteins or receptors coupled to channel-forming proteins via the measured electrical characteristics of the channel-forming proteins.

Unfortunately, several major limitations have prevented patch-clamp technology from revolutionizing receptor science and pharmaceutical drug development. For example, to produce high quality results, the patch-clamp method requires a tremendous effort in technical installation and highly qualified operators. Moreover, in addition to being expensive, a standard patch-clamp setup may require a long set-up time and have a high failure rate.

Thus, there is a need for a system for positioning and/or analyzing cells that is rapid, facile, and suitable for multiarray analysis, such as the system provided by the invention.

Summary of the Invention

The invention provides systems for positioning and/or analyzing samples such as cells, vesicles, cellular organelles, and fragments, derivatives, and mixtures thereof, for electrical and/or optical analysis, especially relating to the presence and/or activity of ion channels.

Brief Description of the Drawings

Figure 1 is a schematic view of a system for positioning and/or analyzing samples in accordance with aspects of the invention.

Figure 2 is a cross-sectional side view of a substrate chip prepared from Si/SiO_2 in accordance with aspects of the invention.

Figure 3 is a cross-sectional side view of a measurement system having planar electrodes in accordance with aspects of the invention.

Figure 4 is a cross-sectional side view of a measurement system having point or wire electrodes in accordance with aspects of the invention.

Figure 5 is a cross-sectional side view of a measurement system having open fluid compartments in accordance with aspects of the invention.

Figure 6 is a top view of a measurement system having multiple measurement sites in accordance with aspects of the invention.

Figure 7 is a cross-sectional side view of the measurement system of Figure 6, taken generally along line 7-7 in Figure 6.

Figure 8 is a cross-sectional side view of a measurement system having optical measurement aids in accordance with aspects of the invention.

Figure 9 is a partially cross-sectional partially schematic side view of a measurement system for combined electrical/optical detection.

Figure 10 is a contour plot of the electric potential adjacent an aperture in a substrate in accordance with aspects of the invention, computed by finite element method (FEM) simulation.

Figure 11 is a plot of current versus time showing a decrease in current upon addition of Ca^{2+} to a final concentration of 4 mM following the docking of vesicles at a 7-μm aperture in the unmodified surface of a suitable substrate.

Figure 12AB is a pair of plots of current versus time showing the time course of vesicle binding and the subsequent development of membranes with very high electrical insulation resistance for (A) a 4-μm aperture and (B) a 7-μm aperture in a poly-L-lysine-coated SiO_2 substrate.

Figure 13 is a plot of current versus time showing the passage of individual vesicles through a 7-μm aperture, as reflected in fluctuations in the plot recorded at a constant clamp voltage, V_c, of –80 mV.

Figure 14 is a plot of current versus time showing the time- and voltage-dependent switching of alamethicin pores in a membrane produced on the substrate ($C_{alamethicin}$ = 0.1 μg/mL in 85 mM KCl) at negative potentials.

Figure 15AB is a pair of plots of current versus time showing the changes in measured membrane resistance of a membrane produced on a Si/SiO_2 carrier chip after fusion with vesicles containing nAChR (nicotinic acetylcholine receptor). Figure 15A shows the membrane resistance during accidental receptor openings in the absence of ligands at 400 mM KCl and positive potentials. Figure 15B shows the membrane resistance 150 seconds after the addition of the nAChR agonist carbamylcholine (20 μm final concentration), where no receptor openings are observed due to desensitization of the receptors.

Figure 16 is a plot of current versus time showing the time course of positioning, binding, and subsequent development of a tight electrical seal for a Jurkat cell.

Figure 17 is a series of plots of current versus time showing the current flowing through the membrane of a Jurkat cell for the indicated positive and negative clamp voltages.

Figure 18 is an analysis of the current flowing through the membrane of a Jurkat cell for a +60 mV clamp voltage showing (A) a representative plot of current versus time, and (B) a histogram showing the relative likelihood of the measured currents.

Detailed Description

The invention provides systems, such as single or multiaperture biochips, for positioning and/or analyzing membrane-bound samples, such as cells, vesicles, cellular organelles, and/or portions thereof. Positioning a sample, as used here, generally comprises locating or placing the sample at a preselected position, within the system, typically for subsequent analysis. Analyzing the sample generally comprises detecting a

presence or activity within the sample, while it is positioned at the preselected position, typically relating at least in part to electrical properties of the sample.

Figure 1 shows a representative system 30 for positioning and/or analyzing samples in accordance with aspects of the invention. The system includes a substrate 32, at least two fluid compartments 34a,b, and at least two electrodes 36a,b. (In some cases, for example, where only optical measurements are to be performed, the system may have only a single fluid compartment and no electrodes.) The substrate comprises a separating wall of electrically isolating material, and may include an aperture and/or window 38 and an associated adhesion surface 40 adjacent the aperture to which samples 42 may be bind or be fixed using any suitable mechanism. The fluid compartments generally comprise any region or volume adapted to support a fluid adjacent the aperture. The electrodes generally comprise any mechanism for creating an electric potential and associated electric field across the aperture. In most embodiments, a first side of the substrate is used as a sample or measurement side or recording site, and a second side of the substrate is used as a reference side, although these roles may be interchangeable. The measurement side is used to hold samples during positioning and/or analysis, and typically includes adhesion surface 40, a measurement fluid compartment 34a and a measurement electrode 36a. The reference side is used to complete the electric circuit, and typically includes a reference fluid compartment 34b and a reference electrode 36b. Generally, despite their different names, the measurement electrode and the reference electrode independently may be set to any suitable voltage, including ground.

The system may include or be interfaced with one or more auxiliary systems, including (1) a sample handling system 44 for introducing, removing, and/or otherwise manipulating fluids and/or samples, (2) an analysis system 46 for analyzing samples, particularly by mechanisms other than direct electrical measurement, and/or (3) an incubation system for storing samples before and/or during assays, and/or (4) a cleaning system for cleaning substrates and/or other system components.

The system may be used for a variety of applications. These applications may include automated and/or high-throughput patch-clamp analysis (e.g., for drug screening), portable biosensor analysis (e.g., for environmental analytes), and so on. These applications also may include the separation of cells or vesicles, the analysis of the sizes of cells or vesicles, the direct functional analysis of ionotropic membrane proteins, for example, in ligand binding studies, and/or the positioning of cells or vesicles for any suitable purpose, including purely optical investigations and/or microinjections, among others. Typically, a sample such as a cell or vesicle is introduced into the measurement compartment and then is directed toward the aperture, for example, using an electric force created by the two electrodes. The sample contacts the adhesion surface, binds across the aperture, and forms an electrical seal with the aperture sufficient for performing an assay of interest. The effects of an applied voltage created by the electrodes then may be studied, typically before and/or after exposure to a suitable assay condition. The studies may be performed by measuring changes in electrical properties across the aperture, such as current, resistance, or the like, and/or by measuring other changes in the sample, such as ion levels or the like.

The assay condition generally comprises any change of condition, optionally including a change in environmental condition, such as sample temperature, but more typically including the addition of one or more reagents such as candidate drug compounds to the sample. The reagent may be a chemical reagent, such as an acid, a base, a metal ion, an organic solvent, or other substance intended to effect a chemical change in the sample. Alternatively, the reagent may have or be suspected to have a biological activity or type of interaction with a given biomolecule. Selected assay components may include membrane-active substances, such as pore promoters, proteoliposomes, and/or membrane proteins. Selected assay reagents also may include oligonucleotides, nucleic acid polymers, peptides, proteins, drugs, and other biologically active molecules.

The system may have one or more advantages over prior systems for measuring electrical properties of cells and vesicles. First, the system is relatively simple, both in the production of electrically insulating patch membranes and in the associated measurements. Thus, the system, alone or in combination with modern microtechnological methods, is suitable for use in automated and/or "high throughput screening" (HTS) applications. Second, the positioning and measuring capabilities of the system are well suited to the combination of electrical and optical measurements, through which, on these planar membranes, obtained by means of the positioning process according to the invention, new, important information concerning membrane channels and receptors may be obtained.

The following sections describe various components and functionalities of the system, including (A) the substrate, (B) the apertures and windows, (C) the adhesion surfaces, including mechanisms for achieving binding, (D) the fluid compartments, (E) the electrodes, (F) multiaperture systems, (G) the analysis system, (H) the sample handling system, (I) the samples, (J) the sample positioning process, and (K) the measurement process, among others.

A. Substrates

The substrate generally comprises any surface or set of surfaces capable of separating two fluid compartments. The substrate typically includes an aperture that passes through the substrate to connect the two fluid compartments and at least one adhesion surface adjacent the aperture for binding a sample such as a cell or vesicle for analysis. The substrate preferably is nonconductive (e.g., electrically insulating), thus reducing or eliminating electrical contact between the two fluid compartments, except through the aperture.

The substrate may be formed of any suitable material. Preferably, the substrate is nonconductive, inert (in the system), and no more than slightly modifiable chemically. Exemplary materials include silicon (including silicon (Si) and silicon derivatives, such as silicon oxide (SO_2; silica) and silicon oxinitride (SiO_xN_y)), glass, quartz, plastic, and so on. Among these, the silicon-based substrates have several advantages. First, they are commercially available. Second, they are easily processed, for example, so that they may be provided with an aperture and/or window, as described below in Example 1. Third, they readily may be coated or otherwise partially or completely covered with insulating

and/or adhesion-promoting materials. Such surface layers include layers of quartz, glass, and solid and/or gelatinous polymers, among others. Such surface layers also include plastomers and elastomers, such as polyimides, polymethylmethacrylates, polycarbonates, and silica gels (e.g. Sylgard). Such surface layers also may be homogeneous or inhomogeneous, where, for example, in the latter case, they may be applied as droplets.

The substrate may include two or more pieces or components, for example, being constructed of a holder on which the material actually relevant to membrane positioning and membrane binding is fastened or into which this material is admitted, where this material for the positioning, or alternatively the binding, of the membranes has at least one aperture.

The substrate may be formed with any suitable geometry, subject to the above limitations. However, preferably, the substrate is at least substantially planar, and more preferably, the substrate is microscopically flat and molecularly relatively planar, particularly at the adhesion surface.

Examples 1-7 below, among others, describe exemplary substrates, including materials, geometries, and relationships with other components of the system.

B. Apertures and Windows

The aperture and window(s) are the portions of the substrate most immediately involved in the positioning and/or analysis of samples.

The aperture generally comprises any opening or other passage through the substrate. This opening may include a hole, a gap, and/or a slit, among others, and may

allow fluid contact between fluid compartments positioned at opposite sides of the aperture. The aperture may be capable of forming an electrical seal with a sample such as a cell or vesicle that is sufficiently "tight" to use in a patch clamp experiment. Exemplary seals (depending on sample type and condition) have included > 10 kΩ, > 100kΩ, > 1 MΩ, > 10 MΩ, > 100 MΩ, > 1 GΩ, > 10 GΩ, >100 GΩ, and even > 1TΩ. Alternatively, or in addition, the aperture may be capable of focusing an electric field with sufficient strength to position a sample such as a cell or vesicle about the aperture. The aperture may include a hole, a gap, and/or a slit, among others.

The aperture is characterized by a length L_{ap} and a diameter d_{ap} The length is determined by the thickness of the substrate adjacent the aperture, generally ranging between about 3 nm and about 1000 μm, and preferably ranging between about 100 nm and 20 μm . The diameter of the aperture, as measured immediately adjacent the binding surface, is influenced by a variety of different factors, which may urge toward either smaller or larger apertures. First, smaller apertures generally increase the quality of the electrical seal between the aperture and the sample, up to a limit. In particular, to form a tight electrical seal, the aperture should be smaller, preferably significantly smaller, than the size of the sample (i.e., $d_{ap} << d_{cell}, d_{vesicle}$), but larger, preferably significantly larger, than the lipids and other molecules present in the sample. Second, smaller apertures generally increase the mechanical stability of the membrane across the aperture. In particular, the force required to deflect a portion of membrane is inversely proportional to the square of the radius of the portion being deflected (i.e., proportional to the value of r_M^{-2}; see, e.g., Example 12), so that the selection of small (e.g., $d_{ap} < 5$ μm) apertures may

significantly increase membrane stability, particularly relative to the typical (e.g., d_{ap} > 100 μm) apertures used in conventional black lipid membrane (BLM) systems. Third, smaller apertures generally increase the strength and focus of the electric field passing through the aperture, which is especially useful when positioning samples. Fourth, larger apertures generally reduce the access resistance, improving the quality of the voltage (or current) clamp, probably by easing the physical access of the conduction ions. Based on these factors, the diameter of the aperture generally is less than about 15 to 20 μm, usually is less than about 10 μm, preferably is less than about 7 μm, and more preferably is less than about 5 μm. In particular, sizes between about 0.3 μm and about 7 μm may yield an outstanding probability and quality of sealing. Stated alternatively, the aperture preferably should have a diameter of no more than a few tens of percent, and more preferably no more than about 30 percent, of the sample diameter. Thus, for cellular samples, which typically have a diameter of greater than about 20 μm, the aperture preferably should have a diameter of no more than about 5-7 μm.

The window generally comprises a portion of the fluid compartment adjacent and providing access to the aperture. The preferred size of the window is determined by factors analogous to those described above for determining the preferred size of the aperture. In brief, the diameter of the window preferably is less than about 1000 μm, and more preferably is significantly smaller, being not more than 100 μm.

Examples 1-7 below, among others, describe exemplary apertures and windows, including geometries and relationships with other components of the system.

C. **Binding and Adhesion Surfaces**

The adhesion surface generally comprises any surface or set of surfaces adjacent the aperture to which samples such as cells and vesicles may bind for analysis. The adhesion surface typically is at least substantially planar over an area exceeding that of the bound portion of the sample but in some cases may be at least slightly concave in the direction of the sample. Thus, the adhesion surface may have an area of at least about 25 μm^2 for a cell that is about 5 μm in (bound) diameter, at least about 100 μm^2 for a cell that is about 10 μm in (bound) diameter, and so on.

Binding, as used here, generally comprises any stable or semi-stable association between a sample and an adhesion surface that results in an electrical seal between the sample and one or more apertures that is sufficiently "tight" to allow the desired measurement. Binding may be mediated by any suitable mechanism, direct or indirect, including electrostatic interactions, covalent bonding, ionic bonding, hydrogen bonding, van der Waals interactions, and/or hydrophobic-hydrophilic[v] interactions, among others. In general, binding may be facilitated by the appropriate selection, treatment, and/or modification of the substrate, the sample, the measurement medium, or a suitable combination thereof.

Binding may be facilitated by appropriate selection of the substrate. Thus, preferred substrates typically include a relatively flat or gently contoured binding surface adjacent the aperture of interest, so that cells or vesicles may bind to form an acceptable seal with the aperture without unwanted or unnecessary deformation. Moreover, preferred

substrates also typically include a modifiable binding surface, so that the surface may be treated as desired to promote binding.

Binding also may be facilitated by appropriate treatment of the substrate, as suggested above. Thus, in some applications, the surface may be treated or otherwise modified so that electrostatic or, in given cases, hydrophobic, van der Waals or covalent binding of vesicles or cells, or -the corresponding membranes or membrane fragments, is promoted. For example, the binding surface may be coated with an adhesion promoter, such as poly-L-lysine, poly-D-lysine, gelatin, collagen, laminin, fibronectin, proteoglycans, polyethylenimine, albumen, BIOMATRIX EHS (Nunc Nalge International), BIOBOND (Electron Microscopy Services, Inc.), and/or MATRIGEL (Becton-Dickinson), among others. Alternatively, or in addition, the binding surface is modified in a way that promotes molecule-specific binding, such as with avidin and/or biotin, or by modification with immobilized lectins. Alternatively, or in addition, the binding surface (especially a silicon binding surface) may be coated with an oxide or oxynitride layer. Alternatively, or in addition, the binding surface may be coated with largely hydrophobic compounds such as Tocopherol. In some embodiments, an electrically charged surface may be generated by modification, in particular, by means of polycations and/or silanes, for example, aminosilanes, or the substrate may have a coating or other surface layer with an electrically charged surface. Microstructured silicon/silicon oxide or silicon/silicon nitride substrates are especially suitable for providing a good electrostatic attraction, after being coated with a substance lending the desired surface charge.[vi] Finally, to improve the quality and consistency of the surface

characteristics, the substrate may be subjected to oxygen plasma cleaned and/or partially or completely hydrophylized before the modification of its surface and/or before its immediate use, in addition to or in lieu of the above modifications. In some aspects of the invention, unwanted hydrophylization/modification of the hydrophobic surface can be avoided by using silicon nitride for the surface layer.

Binding also may be facilitated by appropriate selection and/or treatment of the sample itself. Thus, the sample may include unsaturated lipids or other compositions that increase the fluidity of its membrane, potentially enhancing membrane flexibility during binding and seal formation. Alternatively, or in addition, the sample may include charged lipids or other compositions that increase the charge on the sample, potentially enhancing the ability of the sample to bind electrostatically to substrate surfaces bearing an opposite charge. For example, for a positively charged substrate surface, the vesicle might include negatively charged palmitoyl-oleoyl-phosphatidylglycerol (POPG).

In some cases, binding may be facilitated by interactions between specific binding pairs (SBPs), where one member of the pair is associated with the sample and the other member of the pair is associated with the substrate. The interactions between members of a specific binding pair typically are noncovalent, and the interactions may be readily reversible or essentially irreversible. An exemplary list of suitable specific binding pairs is shown in Table 1.

Table 1: Representative Specific Binding Pairs

First SBP Member	Second SBP Member
antigen	antibody
biotin	avidin or streptavidin
carbohydrate	lectin or carbohydrate receptor
DNA	antisense DNA
enzyme substrate	enzyme
histidine	NTA (nitrilotriacetic acid)
IgG	protein A or protein G
RNA	antisense RNA

Binding also may be facilitated by appropriate selection and/or treatment of the measurement medium. For example, the medium may include binding mediators that participate in or otherwise promote interactions between the sample and substrate, for example, by forming cross-bridges between the sample and substrate and/or by counteracting the effects of binding inhibitors associated with the sample, substrate, or medium. The binding mediators may act specifically, for example, by binding to specific groups or molecules on the sample or substrate. Thus, biotin might act as a specific binding mediator by binding to and cross-linking avidin or streptavidin on the sample and substrate. The binding mediators also may act less specifically, or nonspecifically, for example, by binding to classes or categories of groups or molecules on the sample or substrate. Thus, Ca^{2+} ions might act as a relatively nonspecific binding mediator by binding to and cross-linking negative charges on the sample and substrate. Ca^{2+} ions are particularly appropriate for mediating the binding of cells or vesicles containing negative lipids and substrates containing negative surface charges, such as SiO_2 substrates.

After binding, samples such as cells or vesicles may be broken up, for example, by treatment with a hypotonic medium, such as pure water.

Examples 1-7, 10, and 11 below, among others, describe exemplary adhesion surfaces, including materials, treatments, modifications, geometries, and the kinetics and efficacy of sample binding.

D. Fluid Compartments

The fluid compartments generally comprise any region or volume adapted to support a fluid adjacent the aperture. The compartments may perform several functions, including covering the sample, providing a medium through which the cell may be moved during positioning, and/or providing a medium for establishing electrical contact between the electrodes, among others. The compartments generally may have any suitable volumes, but they typically have volumes between about 0.1 to 40-100 μL. Thus, assays typically require only a limited amount of sample, facilitating the analysis of effects of precious compounds.

The fluid compartments may be closed or open. A closed compartment comprises a compartment that is at least substantially bounded or delimited on all sides by a wall or other separating layer, exclusive of an input and/or output port. In contrast, an open compartment comprises a compartment that is not bounded on at least one side (i.e., over at least some solid angle) by a wall.

Closed compartments typically are physically confined, i.e., bounded by some combination of the substrate, an electrode, and one or more spacers, being established within voids and channels therein. The spacers are used in many embodiments,

particularly those involving planar electrodes, to establish and maintain the relative positions of the substrate and electrodes. Such spacers typically are formed of an electrically isolating material, like the substrate. The spacers may include channels disposed between the aperture and the electrode. These channels, typically filled with a conductive solution, can serve as a sample or reference chamber. It is beneficial if the reference chamber has such small dimensions that the reference buffer solution may be fixed therein by capillary forces, and/or forced therein by surface tension (e.g., at the fluid/air interface in an open compartment).

Open compartments typically are free standing, i.e., not bounded in at least one, typically lateral, direction. Instead, the fluid may be fixed between the substrate and electrode without other physical boundaries by capillary forces and/or surface tension.

Examples 2-7 below, among others, describe exemplary fluid compartments, including geometries, boundaries, and relationships with other components of the system.

E. Electrodes

The electrodes generally comprise any mechanism for creating and/or modulating an electric potential across an aperture, particularly for use in positioning and/or analyzing samples.

The electrodes may be formed of any material capable of inducing current flow through an aperture upon application of a physiological potential. Suitable electrodes include silver, gold, and/or platinum, among others. Preferred electrodes include silver/silver chloride (Ag/AgCl) and/or platinum (Pt) redox electrodes.

The electrodes may be formed with any suitable geometry and be disposed in any suitable arrangement, consistent with their performing their intended function(s). Preferred electrodes have planar, cylindrical, or point geometries. Preferred electrode arrangements are symmetrical, with similar electrodes positioned at similar distances and orientations from each aperture on each side. Symmetrical electrode arrangements generally will create symmetrical electric fields. Typically, the electrodes are located opposite one another across a single aperture, with each electrode reaching into at least one compartment, or at least contacting a surface of it. The electrodes customarily are located at a distance of about 0.5 to 3 mm, and usually about 0.5 to 1 mm, from the substrate, although they can be closer or farther in some embodiments. Depending on embodiment, the electrodes may be attached directly to a recording carrier, or to a cartridge in which this carrier is packaged, and/or to a holder that is not in direct contact to the substrate.

The electrodes should be capable of creating an electric potential sufficient to perform their function, for example, positioning and/or analyzing samples, without unduly disrupting the samples. Preferred electric potentials give rise to electric field intensities of greater than about 100 V/m, particularly adjacent the aperture. In the following materials, one electrode is referred to as a measurement electrode,

Examples 2-7 below, among others, describe exemplary electrodes, including geometries (e.g., planar, cylindrical, and point), materials (e.g., silver and/or platinum), and relationships with other components of the system.

F. Multiaperture Systems

The invention provides multiaperture systems for positioning and/or analyzing samples. These systems include two or more apertures, which may be disposed at the same and/or separate sites. Apertures disposed at the same site may be used to study single samples at two or more positions on the sample. In contrast, apertures disposed at separate sites may be used to study two or more samples, sequentially and/or simultaneously, at one or more positions on each sample. The production of multiaperture systems generally is straightforward, especially using silicon substrates and Ag/AgCl electrodes, both of which are easily microstructurable. In particular, multiaperture systems may be produced from a single continuous substrate having two or more apertures or by joining together two or more smaller substrates each having one or more apertures. The latter approach may be less expensive for substrates such as silicon with costs that increase faster than area.

Exemplary multiaperture systems employ a multiarray layout having a plurality of separate measurement sites. In these systems, each site includes at least one aperture and fluid and electrical contact with at least one fluid compartment and at least one electrode, respectively, on each side of the aperture. The fluid compartments and electrodes on one side of the substrate (the measurement side) generally are separated to allow independent recordings. However, the fluid compartments and electrodes on the other side (the reference side) may be partially or totally combined, because these components typically function merely to provide a common electrical potential (e.g., ground). The sample generally may be positioned on either the measurement or the reference side, although typically it is positioned on the measurement side so that each fluid compartment

independently can contain the same or different types of samples. Thus, in these exemplary systems, several apertures may be used on one substrate, and the measurements may be performed over at least two apertures sequentially and/or in parallel and/or in such a manner that all or several electrodes on one side of the substrate have a common electrical potential, or, alternatively, are combined to form one electrode. Similarly, more than two electrodes and more than one aperture can be present in such a way that at least one electrode, for example, a reference electrode, serves the measurement via more than one aperture, or the measurement arrangement can have a substrate with more than one aperture and twice as many electrodes as apertures in such a way that one aperture always is located between two electrodes.

Measurement sites may be separated using any suitable mechanism, including hydrophilic/hydrophobic surface patterning, as described below, and/or dividing the carrier surface into small compartment wells (e.g., by laminating a thin polydimethylsiloxane (PDMS) layer containing small holes to the carrier surface adjacent the aperture).

The multiaperture system generally may include any number of measurement sites, positioned in any suitable arrangement, with any suitable size or footprint, all consistent with forming electric fields within each site to position and/or analyze samples. Preferred configurations may be selected based on utility and/or convenience. Thus, preferred systems may include features selected from standard microplates, so that the system may be used with standard microplate equipment, including handlers, washers, and/or readers, among others. These features may include a rectangular frame,

with a major dimension of about 125-130 mm, a minor dimension of about 80-90 mm, and a height of about 5-15 mm, although other dimensions are possible. The frame may include a base configured to facilitate handling and/or stacking, and/or a notch configured to facilitate receiving a cover. These features also may include 96, 384, 864, 1536, 3456, or 9600 measurement sites, among others, positioned on a rectangular or hexagonal array. Three exemplary configurations that will fit as rectangular arrays within a microplate-sized frame are listed in the following table:

Number of Sites	Arrangement of Sites	Pitch (mm) Between Sites	Density (/mm^2) of Sites
96	8 × 12	9	1/81
384	16 × 24	4.5	4/81
1536	32 × 48	2.25	16/81

Here, pitch is the center-to-center site-to-site spacing, and density is the number of sites per unit area. These features also may include the color of system components, particularly components in the optical path in optical assays. For example, in fluorescence applications, system components preferably are made of opaque black plastic to reduce background photoluminescence and/or "crosstalk," where crosstalk is the transmission of light emitted in one site to adjacent sites where it may be detected. In contrast, in chemiluminescence applications, system components preferably are made of opaque white plastic to increase reflection of emitted light out of the site by the white surfaces while still reducing crosstalk.

Examples 5 and 6 below, among others, describe exemplary multiaperture positioning and/or analysis systems, including additional features such as reference fiducials not described above.

G. Analysis system

The positioning and measurement system of the present invention optionally may be coupled to or integrated with an analysis system for analyzing samples and sample components. The analysis system generally comprises any mechanism for analyzing or otherwise characterizing samples, qualitatively or quantitatively, other than by direct electrical measurement as used by the positioning and measurement system. The analysis system may require that the sample be separated from the measurement system and/or from other sample components, as described above. Alternatively, or in addition, the analysis system may allow the sample to be studied *in situ*, without such separation. Generally, measurements made by the positioning and measurement system and measurements made by the analysis system may be performed simultaneously or sequentially, in any order or in any combination, in association with or independent of one another.

The analysis system may be based on any suitable analytical technique, including spectroscopic, hydrodynamic, and imaging methods, among others, particularly those adaptable to high-throughput analysis of multiple samples. Preferred analysis systems are based on the optical analysis of samples, particularly luminescence-based optical analysis, but also absorption, scattering, circular dichroism, optical rotation, and imaging, among others. In luminescence analysis, light transmitted from the sample is detected and

analyzed, and properties of the detected light are used to infer properties of the sample, including the presence, size, shape, mobility, quantity, activity, and/or association state of selected components of the sample. In photoluminescence, including fluorescence and phosphorescence, the emission of light from the sample is induced by illuminating the sample with appropriate excitation light. In chemiluminescence, the emission of light from the sample is induced by chemical reactions occurring within the sample. The analysis may involve measuring various properties of the detected light, including its intensity, lifetime, polarization, quantum yield, and Stokes' shift, among others. The analysis also may involve using one or more of these properties in techniques such as fluorescence intensity (FLINT), fluorescence polarization (FP), fluorescence resonance energy transfer (FRET), fluorescence lifetime (FLT), total internal reflection fluorescence (TIRF), fluorescence correlation spectroscopy (FCS), fluorescence recovery after photobleaching (FRAP), and fluorescence imaging, including confocal CCD observation, among others.

In luminescence assays, light typically is detected from a luminophore, i.e., a molecule or other species that emits luminescence. The luminophore may be endogenous or exogenous. Moreover, the luminophore may be the material of interest in the assay but more commonly is simply a reporter that provides information about another material that is the true material of interest. In particular, the luminophore may be an exogenous molecule that reports on (1) membrane potential, (2) the presence or concentration of a target metal, such as Ca^{2+}, Mg^{2+}, and Zn^{2+}, (3) the presence or concentration of an inorganic ion, such as Na^{+}, K^{+}, and Cl^{-}, (4) pH, (5) reactive oxygen species, including

nitric oxide, (6) ion channels, including Ca^{2+} channels, Na^{+} channels, K^{+} channels, and Cl^{-} channels, (7) signal transduction, (8) cell viability, and (9) endocytosis and exocytosis, among others. Suitable luminophores for reporting on this and other information are described in Richard P. Haugland, *Handbook of Fluorescent Probes and Research Chemicals* (6^{th} ed. 1996), which is incorporated herein by reference.

The combination of optical technologies with the patch-clamp technologies presented here permits, for the first time, the distinction or resolution of ligand binding events and channel activities, among others. In this way, for example, important information regarding the stabilization of changes in receptor conformation through ligand binding and/or the functional variation in ligand binding sites in receptors may be obtained.[vii] Such information is potentially important for understanding the particular mode of action of individual agonists and antagonists, and thus exhibits great promise for future drug development.

The optical analysis system typically will include a light source, a detector, and one or more optical relay structures for directing excitation light from the light source to the sample and for directing emission light from the sample to the detector. However, the light source and excitation optical relay structure are optional during analysis utilizing chemiluminescence methods. The optical analysis system may use epi- and/or trans-detection schemes, involving illuminating off of and/or through the sample, respectively.

Exemplary optical analysis systems, and components thereof, are described below under Examples and in the various patents, patent applications, and other materials listed above under Cross-References and incorporated herein by reference. Preferred optical

analysis systems are described in the following materials: U.S. Patent No. 5,355,215, issued October 11, 1994; U.S. Patent No. 6,097,025, issued August 1, 2000; U.S. Provisional Patent Application Serial No. 60/267,639, filed February 10, 2001; and Joseph R. Lakowicz, *Principles of Fluorescence Spectroscopy* (2nd ed. 1999).

The combined system is suitable for simultaneous and/or sequential electrical and optical (e.g., photoluminescence) measurements. Suitable apparatus may include a planar, and vertically easily realizable, optically transparent structure, for example, with the use of planar pointed electrodes, or alternatively point electrodes disposed outside of the vertical lines going through the aperture. This allows not only fluorescence or electrical analysis of single cells (or membranes) but also the combined optical-electrical observation of cells (or membranes), particularly in response to exposure to externally applied substances, such as potential medical drugs. This revolutionary approach allows significantly more efficient selection of new drugs and the elucidation of their molecular action.

Example 7 below, among others, describes an exemplary analysis system. Additional examples are described in the various patents and patent applications listed above under Cross-References and incorporated herein by reference.

H. Sample-handling system

The positioning and measurement system according to the invention optionally may be coupled to or integrated with a sample-handling system for adding, manipulating, exchanging, and/or removing samples and sample components, including cells and vesicles, sample media, and compounds and reagents, such as candidate modulators

and/or other analytes. The sample-handling system may add samples such as cells or vesicles to arbitrary compartments, convey liquid into and/or out of arbitrary compartments, and/or exchange samples and/or liquid between arbitrary compartments, among others. The sample-handling system also may separate samples, or sample components, in particular using capillary electrophoresis (CE) and/or high-pressure liquid chromatography (HPLC), and serve the analysis of the separated substances, or it can be provided with means that serve the continuous or regular testing of the state of the liquid in the compartments as well as with means for retroactive regulation according to preset filling parameters. Because it is reasonable, according to the analysis strived for, to bring the membrane into contact with measurement solution on both sides, the addition of a substance to be investigated obviously can be done on the side customarily serving as the reference side. The sample-handling system may be multiplexed to interact with several substrates and/or with a multiaperture substrate, among others.

The sample-handling system may be based on any suitable mechanism, including tubes, pumps, hydrostatic pressure differentials, electro-osmotic processes, piezo drop-on-demand processes, ink-jet processes, contact transfer processes, temperature-controlled processes, and/or mechanical displacement, among others. In some embodiments, fluids such as reference buffers may be introduced into a pasty gel, whereby an exchange of the liquid lying outside the gel is possible without changing the composition of the reference buffer stored in the gel. Suitable gels include agarose and polyacrylamide.

Example 2 below, among others, describes an exemplary sample handling system. Additional examples are described in the various patents and patent applications listed above under Cross-References and incorporated herein by reference, including U.S. Patent Application Serial No. 09/777,343, filed February 5, 2001; and U.S. Provisional Patent Application Serial No. 60/267,639, filed February 10, 2001.

I. Samples

The sample generally comprises any species having a membrane or other surface capable of forming a seal with an aperture sufficient for performing electrical measurements such as patch clamp experiments. The sample may include cells, vesicles, cellular organelles, membrane-bound viruses, and fragments, derivatives, and mixtures thereof.

Biological samples may include or be derived from (1) eukaryotic cells, i.e., cells with a nucleus, including cells from plants, animals, fungi, yeast, and protozoans, or enucleated derivatives thereof; (2) prokaryotic organisms, including bacteria and archaebacteria; (3) viruses; (4) organelles or extracts, such as nuclei, mitochondria, endosomes, the Golgi apparatus, peroxisomes, lysosomes, endoplasmic reticulum, chloroplasts, axons, and dendritic processes, among others; and (5) gametes, including eggs and sperm. These cells and other materials may be obtained from any suitable source, including cell cultures, patient samples, and tissues, among others. These cells also may be subjected to any suitable treatments to alter membrane properties, for example, to introduce a novel or modified ion channel, among others. These treatments may include genetic modification by any suitable method, including chemical treatment, irradiation, transfection, infection, and/or injection, among others.

Vesicles and other synthetic samples may include or be derived from (1) unilamellar vesicles, (2) multilamellar vesicles, (3) small vesicles (having diameters less than about 1000 nm), (4) large vesicles (having diameters greater than about 1000 nm), (5) monodisperse vesicles, and (6) polydisperse vesicles. These vesicles may be formed from any suitable lipid(s) and/or protein(s) using any suitable technique. Exemplary lipids include DLPC, DMPC, DPPC, DSPC, DOPC, DMPE, DPPE, DOPE, DMPA, DPPA, DOPA, DMPG, DPPG, DOPG, DMPS, DPPS, and DOPS, among others.

Examples 8 and 15-17 below, among others, describe exemplary samples, including vesicle and cell samples.

J. Sample positioning

The electrical, optical, and/or other analysis of samples generally is preceded by a positioning step, in which the sample is directed to or otherwise located at the adhesion surface. Sample positioning generally occurs in two sequential substeps: (1) a first (prepositioning) substep, in which the sample is introduced to the measurement compartment, and (2) a second (micropositioning) substep, in which the sample is brought into proximity or actual contact with the adhesion surface. These steps may be performed robotically, at least substantially without direct human involvement or intervention.

The prepositioning substep generally involves introducing the sample to the measurement compartment, preferably in a manner that facilitates subsequent binding of the sample to the adhesion surface. Thus, the sample may be introduced generally above the adhesion surface (or associated aperture), so that it is directly between the electrodes,

if they are symmetrically arranged, and so that gravity will tend to pull it straight down toward the aperture. Alternatively, or in addition, the sample may be introduced relatively close to the adhesion surface, and/or with an initial velocity toward the adhesion surface, among others.

The micropositioning step generally involves bringing the sample into proximity or actual contact with the adhesion surface, once the sample is in the measurement compartment. Generally, samples may be micropositioned using any suitable force or other mechanism, including sedimentation (e.g., "1g-sedimentation" under the influence of gravity), electromagnetic forces (e.g., electrophoresis, electro-osmosis, and the like), optical forces (e.g., optical tweezers), fluid-mediated forces (e.g., pressure, vacuum, flow, diffusion, and the like), and/or manual forces. Alternatively, or in addition, the cells may be positioned by fluid flow from the sample compartment to the reference compartment by hydrostatic pressure difference or by difference in surface tension between the two compartments.

Preferably, samples are micropositioned using electromagnetic forces, specifically, field focusing of an electric field created by applying a potential across the two electrodes. In brief, this technique exploits the field focusing that occurs adjacent the aperture, creating an electric force that, at least adjacent the aperture, points in all positions toward the aperture, with a strength that increases with proximity to the aperture.

Examples 2 and 9 below, among others, describe exemplary methods for prepositioning and micropositioning samples, respectively.

K. Measurement Process

The measurement process provided by aspects of the invention allows in particular the measurement of ion channel flows in a reliable and reproducible manner, often with a high signal-to-noise ratio. These abilities reflect the precise positioning and electrically tight binding of cells, vesicles, cellular organelles, and/or membranes of corresponding origin, at microstructured apertures in a planar substrate. This electrically tight binding may be achieved at least in part by strong interactions between the surface of the substrate and the surface of the bound membrane, such as strong electrostatic attractions.

The electrical characteristics of transmembrane ion channels or ionotropic receptors may be characterized using "voltage-clamp technologies," such as classical voltage-clamp, patch-clamp, and oocyte voltage-clamp, among others.[viii] Specifically, an electrical potential difference is applied across the membrane containing the relevant ion channel(s), and, simultaneously, the current necessary to maintain this difference is analyzed. The relationship between the voltage and current may be expressed mathematically using Ohm's Law, which states that $V = IR$, or equivalently, $I = V/R$, where V = voltage, I = current, and R = resistance. The current provides insight into membrane electrical properties, such as its conductivity, and therefore insight into the conformation state of the channel-forming protein (e.g., open (passing ions) or closed (blocking ions)). Thus, the current may be used to analyze voltage dependencies, ligand binding events, and so on.

The ability of current measurements to yield meaningful data in a patch clamp or other electrophysiology experiment is dependent on ensuring that the measured current

reflects ion flow through the sample (e.g., through ion channels in the membrane) and not through other components of the system. In particular, to obtain acceptable or better signal-to-noise ratios, it typically is desirable to ensure that the measured current includes no more than a ten or twenty percent contribution from unintended sources (e.g., that sources of noise lie under the signals to be measure by approximately the factor of five or ten). Unfortunately, the ion flow through ionotropic membrane proteins with 0.1 to 50 pA at a –60-mV membrane potential is in general very small, so that leakage currents occurring essentially between the membrane and its fastening quickly may become significant, representing a principal problem in all voltage-clamp technologies.

The problem of leakage currents can be solved in a variety of ways. For example, enlarging the aperture and thereby the patch of membrane to be analyzed may reduce the contribution of leakage currents to the total signal, because the area of the membrane patch and so the intended signal will grow as the radius of the aperture squared, while the circumference of the membrane and so the leakage current will grow merely as the radius. Unfortunately, increasing the size of the membrane may lead to a loss in specificity, particularly in biological systems, because more channels and more types of channels will be in the membrane area analyzed. Then, in general, an unambiguous or completely artifact-free statement, for example, in the case of the addition of ligands, may no longer be possible.

Establishing and maintaining a very high seal between the membrane and aperture also may reduce the contribution of leakage currents. This invention uses this principle, at least in part. To implement the seal, a planar substrate chip having a surface that is

strongly adhesive for cells and vesicles is used. This chip separates the two compartments clamped at different potentials during the measurement, where a (sub)micrometer-sized aperture is located in its middle. This aperture is filled with reference buffer solution and electrically tightly sealed during current measurements by strong binding of cells and vesicles to the surface. This electrically tight binding may permit the measurement of very small ion flows (e.g., down to at least about 0.1 pA) and, concomitantly, the plotting of membrane resistance with a good or better signal-to-noise ratio.

Further (mechanical) stability may be derived from using capillary forces to fill and store the reference and/or measurement buffers. In particular, unbounded or open fluid compartments may experience fewer disturbances (e.g., due to temperature differentials) of the membrane due to hydrostatic pressure than closed systems.

The measurement systems described here may be used for a variety of applications, some of which are described below in Example 16, including "perforated-patch," "whole-cell," and "inside-out" patch clamp techniques. For example, measurement systems of the planar type are particularly well suited, due to the short diffusion times associated therewith, to the use of "perforated-patch" techniques.[ix] In these techniques, an electrical connection to the interior of the cells (cytosol) is achieved by permeabilization of the area of the membrane suspended across the aperture with pore-forming antibiotics. An advantage of this technique is that it does not require washing out the cytosol with measurement buffer solution for simultaneous electrical access. In particular, a pore former such as, for example, amphotericin B or nystatin can be added to the reference compartment, after a biological cell, or, under special

circumstances, a vesicle (if its mechanical stability is sufficiently high) is bound to the upper side of the aperture. In doing so, the rate of perforation of the membrane patch over the aperture is significantly greater than in comparable standard patch-clamp techniques.

For example, measurement systems of the planar type also are particularly well suited to the use of "whole-cell" techniques. In these techniques, an electrical connection to the cytosol is achieved by destroying the membrane patch, for example, using a voltage pulse. This destruction, in turn, may facilitate the simple addition of larger proteins into the cytoplasm, via the reference solution, again because the planar layout of the measurement system allows significantly faster diffusion of large macromolecules into the cytosol or the interior of a vesicle than comparable standard whole-cell techniques.[x]

The system facilitates the addition and/or exchange of various system components, including solutions and/or substances, as suggested above. For example, in some applications, the measurement solution, the reference solution, or both solutions may be replaced by another solution. Alternatively, or in addition, a substance to be analyzed may be added to the solution on the measurement and/or reference side. The substance may include a pore former that can be added to one or both compartments with the aim of increasing the electrical conductivity, or, alternatively, the permeability of the membrane with respect to certain ions. The substance also may include detergent-solubilized proteins or proteoliposomes of arbitrary size, with the aim of fusing them to the membrane over the aperture and thereby making arbitrary membrane proteins contained therein accessible to electrical or optical measurements. The fusion of proteoliposomes is

described in detail in U.S. Patent Application Serial No. _____, filed September 14, 2001, titled EFFICIENT METHODS FOR THE ANALYSIS OF ION CHANNEL PROTEINS, and naming Christian Schmidt as inventor.

Examples 12-16 below, among others, describe exemplary results obtained from various electrical measurements on vesicle and cell-derived membranes.

Examples

The following examples describe selected aspects and embodiments of the invention. These examples are included for illustration and should not be interpreted as restricting, limiting, or defining the entire scope of the invention. Additional examples are described in the following patent applications, which are incorporated herein by reference: U.S. Provisional Patent Application Serial No. _____, filed September 13, 2001, titled HIGH-THROUGHPUT PATCH CLAMP SYSTEM, and naming Christian Schmidt as inventor; and U.S. Patent Application Serial No. _____, filed September 14, 2001, titled EFFICIENT METHODS FOR THE ANALYSIS OF ION CHANNEL PROTEINS, and naming Christian Schmidt as inventor.

Example 1: Substrate Chip

This example, illustrated in Figure 2, describes an exemplary Si/SiO_2 chip substrate 50 for use in positioning and/or studying cells, vesicles, and the like, in accordance with aspects of the invention.

The substrate includes a body 52, a surface layer 54, a window 56, and an aperture 58. The body comprises an at least substantially planar, commercially available silicon wafer. The surface layer comprises a silicon oxide or silicon oxynitride layer formed

adjacent one or more sides of the body. In this embodiment, the surface layer has a thickness of at least about 50 to 200 nm and provides at least one adhesion surface 60a,b capable of binding cells, vesicles, and/or other samples. The window and aperture comprise openings through the body and surface layer, respectively. These openings are at least substantially concentrically aligned, with dimensions sufficient to allow fluid contact between opposite sides of the substrate.

The substrate may be produced using any suitable method, including photolithography or, for apertures having diameters of less than about 1.5 μm, electron beam lithography. These methods may involve anisotropic etching of the silicon in a medium containing KOH, as well as reactive ion etching of the silica layer.

In alternative embodiments, the substrate may include a body and/or a surface layer having a different geometry and/or formed of different materials. In addition, the window may be absent, or the window and the aperture both may be openings in the body, particularly in embodiments lacking a surface layer.

Example 2: Measurement System with Planar Electrodes

This example, illustrated in Figure 3, describes an exemplary measurement system 70 having planar electrodes, in accordance with aspects of the invention.

The measurement system includes a substrate 72, at least two fluid compartments 74a,b, at least two redox electrodes 76a,b, and optionally at least four spacers 78a-d. In this embodiment, all of these components are at least substantially planar; however, in other embodiments, one or more of these components may have a different geometry. Generally, samples may be introduced into either compartment, and measurements may

be performed with the system in any orientation. However, to simplify the description, the top fluid compartment 74a and top electrode 76a (as drawn) are referred to here as the measurement compartment and measurement electrode, and the bottom fluid compartment 74b and the bottom electrode 76b (as drawn) are referred to as the reference compartment and the reference electrode.

The substrate is used to support cells, vesicles, and other samples for electrical analysis. The substrate includes a body 80, a window 82, and an aperture 84 connecting the two fluid compartments. The substrate further includes at least one adhesion surface 86a,b positioned adjacent one or both ends of the aperture for binding cells, vesicles, and/or other samples. An exemplary substrate is described in more detail in Example 1.

The fluid compartments are used to support fluids such as electrolyte solutions or growth media in apposition to the substrate and aperture. The compartments are formed by apertures or voids in the substrate, spacers, and/or electrodes.

The electrodes are used to generate an electric potential and associated electric field across the aperture. Measurement electrode 76a comprises a 0.8-mm thick chlorinated square (e.g., 4×4 mm^2) or annular (e.g., d = 2 mm) silver (Ag) plate, preferably having a frustoconical or funnel-shaped opening 88 (e.g., d_{min} = 0.4 to 1 mm). The measurement electrode is positioned at least substantially parallel to the surface of the substrate, preferably at a distance of up to about 1 mm from the surface of the substrate. The measurement electrode further is positioned so that opening 88 is at least substantially concentrically positioned above aperture 84. Reference electrode 76b comprises a 2-mm thick square silver plate (e.g., 20×20 mm^2), which preferably has a

purity greater than about 99.98% silver. The preferred silver/silver chloride (Ag/AgCl) reference electrode may be produced during manufacture of the system, for example, (1) by exposing the reference compartment to a molecular Cl_2 gas, typically while applying a potential to the electrode, or (2) by filling the reference compartment with 1 M HCl, and then chlorinating the exposed silver for 90 seconds under a 0.8-V potential. The substrate is mounted, after its underside is wetted with buffer solution, over the reference compartment filled with reference buffer solution. In this embodiment, the reference electrode functions to support and maintain other components of the system.

The spacers may be used for several functions, including (1) separating the substrate and electrodes, (2) forming the fluid compartments, and (3) contributing to the structure of a feed opening 90 used to introduce samples to the sample compartment. Specifically, first and second spacers 78a,b are positioned about the measurement electrode. These spacers include openings 92a,b that may be aligned concentrically with opening 88 in the measurement electrode and aperture 84 in the substrate to form a "feed opening" for introducing samples into the system. The feed opening may be of arbitrary form; typically, however, it is elliptical, in particular circular, with a preferred diameter of about 0.2 to 2 mm, and a more preferred diameter of about 0.5 to 1 mm, to facilitate its concentric alignment with the aperture. A third spacer 78c is positioned between the measurement electrode and the substrate to form an insulating barrier between these two elements. This spacer, preferably formed from silicone (e.g., Sylgard 184, Dow Corning, USA), includes a ring-like opening 92c that again may be mounted concentrically about aperture 84, for example, with a radius r of about 1 mm. The ring-like opening forms,

together with the meniscus that forms between the chip and measurement electrode, the sample chamber (sample compartment) for the addition of cells, vesicles, and/or measurement solution. Finally, a fourth spacer 78d is positioned between the reference electrode and the substrate to forming an insulating barrier between these two elements. This spacer, preferably formed as a 0.5 to 2 mm-thick silicon rubber seal (e.g., Sylgard), includes a channel or chamber 92d having dimensions of about 1 mm in width and less than about 6 mm in length. The spacer may be imprinted and, if filled with buffer solution, produce contact between the aperture, or alternatively the membrane, and the reference electrode.

The measurement system may be configured or adapted to facilitate the addition, positioning, and/or analysis of samples. Thus, the setup preferably has means on one or both sides of the substrate that make possible an addition of liquid, a storage of liquid, and, in given cases, an exchange of liquid, as well as the addition of cells, vesicles, or other cellular organelles, or parts of the same, between the substrate and the electrode(s). For example, during measurement, or membrane production, a small (e.g., 5 to 10 μL) volume of measurement or vesicle solution (e.g., a cell suspension) may be added (e.g., by pipette) directly to the feed opening, the window, and/or the aperture, on the measurement side of the substrate or on the upper side of the measurement electrode. The aperture preferably has a diameter such that, when a voltage differential exists over the chip, an inhomogeneous electrical field, mediated by the electrodes, is set up around the aperture. This field may increase in magnitude near the aperture, such that samples can be moved electrophoretically toward the aperture. Furthermore, the substrate preferably

includes at least one surface 94a,b, on one or both sides of the aperture, that is attractive for biological membranes, permitting the molecule-specific and/or multivalent ion-mediated binding of cells, vesicles, membrane fragments, and/or cellular organelles. The surface of the substrate further may be structured to create hydrophilic and hydrophobic areas, with the hydrophilic area preferably positioned around the aperture.

Example 3: Measurement System with Point or Wire Electrodes

This example, illustrated in Figure 4, describes an exemplary measurement system 110 having point or wire electrodes, in accordance with aspects of the invention.

The measurement system includes a substrate 112, two fluid compartments 114a,b, and two point or wire electrodes 116a,b. The substrate and fluid compartments are used to support samples and fluids, respectively, as described above. The substrate includes a body 118, a window 120, and an aperture 122 connecting the two fluid compartments. The substrate further may be surface modified and/or fastened to a holder, including a glass or Teflon holder. The electrodes are used to apply an electric potential and associated electric field across the aperture, also as described above. Here, the electrodes comprise the chlorinated end surfaces 124a,b of two silver wires 126a,b, or, alternatively, two silver electrodes, disposed above and below the substrate. The electrodes preferably have diameters between about 0.1 and 2 mm and a relative separation of about 4 mm. In some embodiments, the electrodes may be provided with a protective outer layer 128a,b that covers and protects the outside surface of the electrodes, except at the end surfaces.

The measurement system may be used for positioning and/or analyzing samples. In an exemplary approach, sample medium is added to both sides of the substrate, and held between the substrate and electrode by capillary forces. Next, the offset is calibrated, and a suitable voltage is applied (typically, V = –60 to –100 mV). Then, cells or vesicles are added to an appropriate (e.g., modified) side of the substrate, and cell binding and/or membrane formation are pursued with the aid of a change in the electrical parameters. Finally, the properties of ion channels or other membrane components are studied using suitable electrophysiology methods. Throughout, the addition or exchange of samples and/or sample media may be performed using a sample handling system, as described above, such as a pipette or tube mounted near the aperture.

Example 4: Measurement System Having Open Fluid Compartments

This example, illustrated in Figure 5, describes an exemplary measurement system 150 having open fluid compartments, in accordance with aspects of the invention.

The measurement system includes a substrate 152, at least two fluid compartments 154a,b, and at least two electrodes 156a,b. These components perform at least substantially the same functions as their namesakes in Examples 2 and 3.

The substrate comprises an insulating silicon chip 158. The substrate may include a groove that is closed by a thin silicon nitride (Si_3N_4) 160 / silicon oxide (SiO_2) 162 diaphragm containing a small aperture 164 having a diameter that usually is less than about 20 μm. The substrate further may include a surrounding insulating layer 166, for example, thermally grown silicon oxide, to reduce system capacitance. The surface of the substrate may be treated to promote the tight adhesion of cell or vesicle-associated lipid

bilayers, for example, by (1) physisorption of poly-L-lysine (with a typical molecular weight greater than about 15,000 daltons), (2) chemical modification with 4-aminobutyl-dimethyl-methoxysilane, and/or (3) attachment of molecules that bind (specifically or nonspecifically) to the cell surface (e.g., lectins), among others.

The fluid compartments comprise open regions of the substrate surface adjacent the aperture to which fluid is confined. Here, fluid is confined by a combination of hydrophilic and hydrophobic interactions. Specifically, fluid is attracted to the region adjacent the aperture by hydrophilic interactions and excluded from regions away from the aperture by surrounding layers of hydrophobic material 168a,b attached or bound to the surface. Consequently, the buffer compartments are delineated by the surface of the substrate on one side and by surface tension on the opposing side, creating dome-shaped compartments, as shown.

The electrodes comprise conductive elements such as Ag/AgCl for generating an electric potential across the aperture. The electrodes, which may be used for positioning and/or recording, are immersed in the fluid compartments. The electrodes may be directly attached to the substrate (e.g., by sputtering or printing) or to a container that contains the substrate. Here, a first (measurement or recording) electrode is used to apply a measurement voltage, and a second (reference) electrode is used to apply a ground.

The measurement system may be used for positioning and/or analyzing samples, at least substantially as described above. In particular, upon application of a voltage between the two fluid compartments, mediated by the redox electrodes immersed in the two compartments, a strongly inhomogeneous field is created around the aperture that

attracts cells, vesicles, and other charged objects towards the aperture. After these samples bind and/or form membranes, they may be analyzed electrically and/or optically, among others.

Example 5: Measurement System Having Multiple Measurement Sites

This example, illustrated in Figures 6 and 7, describes an exemplary measurement system 190 having multiple measurement sites, in accordance with aspects of the invention. The drawings show two alternative embodiments, separated by break lines, that include different carrier/electrode configurations.

The measurement system includes a plurality of measurement sites, each capable of positioning and/or analyzing a sample, as described above. More specifically, the measurement system includes a substrate 192, a plurality of fluid compartments 194a,b, and a plurality of electrodes 196a,b. The measurement sites are formed from portions of the substrate and combinations (e.g., pairs) of fluid compartments and electrodes. The substrate preferably comprises (1) a silicon body 198, (2) a silicon nitride diaphragm 200 having a plurality of apertures 202, at least one per measurement site, and (3) a hydrophobic and/or insulating surface coat 204. The fluid compartments preferably comprise (1) a plurality of measurement compartments 194a, and (2) at least one reference compartment 194b. The electrodes preferably comprise (1) a plurality of measurement electrodes 196a, at least one per aperture or measurement site, and (2) at least one reference (ground) electrode 196b. The measurement system further may include additional features, such as (1) a support or carrier plate 206 to simplify the

design and/or to increase the reliability of the system, and/or (2) one or more reference fiducials 207 for reference and/or alignment purposes, as described below.

The components of the measurement system are described in more detail in subsequent subsections. Briefly, on one side, the substrate contains a patterned surface that physically separates the measurement compartments, allowing independent measurements. The patterned surface may be created by the patterned attachment of hydrophilic materials at measurement sites and hydrophobic materials at intervening positions. The measurement compartments may be accessed independently using a separate measurement electrode for each compartment, where each electrode is connected independently to one or more voltage sources, such as a voltage clamp circuit. On the other side, the substrate contains a reference compartment that can be separated but that preferably is unified to form a single compartment in contact with a single (usually ground) electrode. In some embodiments, the substrate includes a silicon chip containing grooves that are closed by a silicon nitride/silicon oxide diaphragm. The diaphragm includes a small aperture having a diameter of less than about 20 μm. The substrate otherwise may be surrounded by an insulating layer, for example, a thermally grown silicon oxide layer, to reduce the system capacitance.

Substrate

The substrate generally comprises any structure adapted to provide two or more sites for positioning and/or analyzing samples electrically, as described above. The substrate may be formed from any suitable material, including silicon, plastic, and/or glass. The substrate generally may include any number of sample sites arranged in any

suitable format, as described above. Preferred formats include 8 × 12 (96) rectangular arrays and 16 × 24 (384) rectangular arrays, with standard microplate footprints. Some embodiments may include additional sites, including additional rows or columns of sites. For example, in one such embodiment, the system includes an additional row of sites, configured as an 8 × 13 (104) rectangular array.

Fluid compartments

The fluid compartments generally comprise any region adapted to support fluid for bathing the sample and for providing electrical contact between the measurement and reference compartments.

The measurement compartments are used for positioning and/or analyzing samples. These compartments are defined by hydrophilic spots on the chip surface, surrounded by a hydrophobic surface coating, for localizing fluid. The measurement compartments include an aperture positioned within the hydrophilic spot and a measurement electrode positioned for electrical contact with the associated measurement fluid. The hydrophilic spot typically includes an at least substantially planar or concave adhesion surface, which may be selected and/or treated as described above to promote sample binding and/or membrane formation.

The reference compartments are used for completing the electric circuit, typically to electric ground. These "backside" compartments may be combined to form one or more large compartments (corresponding to two or more measurement compartments), since the separate compartments typically would contain the same buffer solution and

each be connected to ground. In particular, a single large backside compartment and a single backside electrode are sufficient for spatial resolution of individual recordings, if the measurement compartments are addressed individually. In some embodiments, the backside electrode may be deposited directly on the recording chip or on an embedding cartridge.

Electrodes

The electrodes generally comprise any mechanism adapted to apply an electric potential across the aperture, with each measurement site in contact with at least one measurement electrode and at least one reference electrode, as described above.

The composition of the electrodes is selected to allow current flow at physiological potentials. Preferred electrodes include silver/silver chloride (Ag/AgCl) and/or platinum (Pt) redox electrodes for both the sample and reference compartments. Particularly preferred electrodes include silver (Ag) as the electrode material, chlorinated within a chlorine (Cl_2) atmosphere.

The number of electrodes may vary. The upper side of the substrate (associated with the measurement compartments) preferably includes enough separate electrodes independently to address each corresponding recording site, e.g., 8 × 13 electrodes on a 2.25 mm grid. In contrast, the lower side of the substrate (associated with the reference compartment) preferably includes a single electrode.

The electrodes may be insulated outside the measurement compartment. Preferred insulation material preferably has a high electrical resistance and a low dielectric constant and loss. Particularly preferred insulation material is produced from Teflon, silicon

nitride (Si_3N_4), and/or Sylgard by spin coating or chemical vapor deposition (CVD). These materials are sufficiently hydrophobic (even after short oxygen-plasma treatment) to confine the measurement and reference compartments. Moreover, these materials may include or be formed to include grooves or holes, potentially improving fluid support and/or reducing evaporation.

The electrodes at the various measurement sites may be connected electrically to corresponding contacts 208 for clean and/or easy access to appropriate electronic components, such as amplifiers, recording devices, and the like. In a preferred embodiment, the contacts are positioned near the edge or border of the substrate, and a bonding wire 210 joins the electrodes and contacts, although more generally any mechanism capable of establishing an electrical connection may be employed. In particular, the electrodes can be bonded to contacts placed on a plastic (e.g., polypropylene) carrier that embeds the entire recording chip.

Support element

The support element generally comprises any mechanism for independently and portably supporting the substrate and associated system components, potentially simplifying design and/or increasing reliability. The support element may support the substrate at its edges and/or in its interior, with the interior support potentially reducing or preventing sagging and/or stress of the substrate. In an exemplary embodiment, the support element includes a carrier plate 206 and a spacer 212 sandwiched between the substrate and the carrier plate near the edges of the substrate. The carrier plate may be formed from glass (e.g., PYREX) and/or any other suitable material. The substrate,

spacer, and carrier plate may be joined using any suitable mechanism, such as anodic bonding. The separation between the substrate and the carrier plate (i.e., the spacer thickness) preferably is chosen to be less than about 1 mm, to allow filling of the backside (i.e., reference) compartment by capillary forces. By extending the glass plate over the borders of the chip, in some embodiments, it may be possible to bond the upper electrodes to contacts placed on the glass plate.

Reference fiducials

The reference fiducials generally comprise any feature or characteristic of the measurement system adapted to provide information that facilitates sample handling and/or analysis, for example, as described in U.S. Patent No. 6,258,326, issued July 10, 2001, which is incorporated herein by reference.

The reference fiducials, or a subset thereof, may be used to encode information and/or to provide reference positions. For example, the reference fiducials may encode information relating to the identity of the manufacturer of the system and/or one or more properties of the system and/or the associated samples. Alternatively, or in addition, the reference fiducials may provide reference positions useful to correct for cross-system drift (due to dimensional irregularities in the system) and/or to align the system with ancillary devices, such as an electrical device for electrical analysis and/or an optical device for optical analysis.

The reference fiducials may encode information using any suitable mechanism, including electrical and/or optical mechanisms. For example, the reference fiducials may encode information electrically, based on the resistance, capacitance, and/or inductance,

among others, of a particular portion or portions of the system. Alternatively, or in addition, the reference fiducials may encode information optically, based on the size, shape, position, color, absorptivity, reflectivity, and/or transmissivity of a particular portion or portions of the system.

The reference fiducials may be identified and read using any suitable mechanism, including the electrical device and/or optical device used in sample analysis.

Example 6: Measurement System Having Optical Measurement Aids

This example, illustrated in Figure 8, describes an exemplary measurement system 230 having optical measurement aids, in accordance with aspects of the invention.

The measurement system includes a substrate 232, a plurality of fluid compartments 234a,b, a plurality of electrodes 236a,b, and a carrier plate 238, at least substantially as described above in Example 5. However, the system further includes an optical measurement aid for use in conjunction with a suitable optical analysis system, as described above. The optical measurement aid generally comprises any element or mechanism adapted to facilitate and/or enable optical analysis of samples such as cells or vesicles positioned on or near the substrate. The optical measurement aid may comprise a modification of one or more of the elements listed above and/or a new element in addition to and/or in lieu of one or more of the elements listed above.

The optical measurement aid may comprise a support element that includes a short spacer and/or a thin, optically transparent carrier plate, as described above. A short spacer and/or a thin carrier plate may shorten the optical path length between the optical device and sample, by reducing the separation between the substrate and carrier plate. A thin

carrier plate also may better match the optical requirements of the optical analysis system. To this end, the thickness of the carrier plate may be selected to correspond to the thickness of a standard microscope cover slip, for example, 0.08 to 0.13 mm thick (No. 0), 0.13 to 0.17 mm thick (No. 1), 0.16 to 0.19 mm thick (No. 1½), or 0.17 to 0.25 mm thick (No. 2), among others. To related ends, the carrier plate may be selected to improve overall optical transmission, for example, by using crystal-clear, pure water-white glass or super clarity, clear-white borosilicate glass. Alternatively, or in addition, the carrier plate may be selected to improve transmission of polarized light, for example, by using strain-free glass or fused silica. Alternatively, or in addition, the carrier plate may be selected to have uniform surface quality, exceptional flatness, and/or precise dimensions, among others.

The optical measurement aid also may comprise a carrier plate or other interface having an array of lenses 240 such as microlenses that correspond in number and spacing to the array of measurement sites. These lenses may be formed from any suitable material (such as glass or plastic) using any suitable technique (such as etching or molding). The lenses may be used for high-magnification and/or high numerical aperture analysis of samples, including the analysis of single cells positioned on single apertures. To assist such analysis, the x-y resolution of the recording apertures and microlenses preferably is less than a few (e.g., about 1-4) μm, which may be facilitated using a high-precision bonding process. Moreover, the z resolution of these components preferably is less than a few (e.g., about 1-4) μm, which may be facilitated using lenses having high numerical apertures.

The lenses in the array generally may have any shape capable of collecting light from the sample and/or focusing light onto the sample. For example, the lenses may be plano-convex, meaning that they have a flat (plano) surface and an opposed outwardly bulging (convex) surface. The plano-convex lenses may have two orientations. In the first orientation, exemplified by lens 240, the convex surface 242 faces toward the sample site, and the planar surface 244 faces away from the sample site. In the second orientation, exemplified in Figure 8 by lens 240', the convex surface 242' faces away from the sample site, and the planar surface 244' faces toward the sample site. In either orientation, the lens will collect light transmitted from the sample and direct the collected light toward a detector, such as an imaging detector (e.g., a charge-coupled device (CCD)) or a point detector (e.g., a photomultiplier tube (PMT)), among others.

The optical measurement aid also may include a window 246 in the substrate having a shape configured to match an optical sensed volume, including the frustoconical shape through which excitation light is directed onto the sample and/or from which emission light is detected from the sample, for example, as described in U.S. Patent Application Serial No. 09/478,819, filed January 5, 2000, which is incorporated herein by reference. The matching may be used in optical analysis to increase sensitivity (for example, by avoiding detection from walls of the sample well) and/or to decrease sample volume, among others.

Example 7: Analysis System

This example, illustrated in Figure 9, describes an exemplary measurement system 270 for combined electrical/optical detection, in accordance with aspects of the invention.

The combined measurement system includes an electrical analysis system 272 and an optical analysis system 274.

The electrical analysis system generally comprises any system for performing electrical measurements such as patch clamp measurements on a sample such as a cell, vesicle, or biological organelle. The electrical analysis system may include any suitable combination of apertures 275, substrates 276, fluid compartments 278a,b, and electrodes (not visible in this view), among other elements, as described above. Exemplary systems are described above in Examples 2-6.

The optical analysis system generally comprises any system for detecting light transmitted from the sample, particularly photoluminescence and chemiluminescence light. The optical analysis system may include a light source 280, a detector 282, and an optical relay structure 284 for transmitting excitation light from the light source to the sample and emission light from the sample to the detector. The system further may include additional components for performing additional and/or duplicative functions, including (1) filters 286 positioned in the excitation and/or emission optical paths for altering the intensity, wavelength, and/or polarization of the excitation and emission light, respectively, (2) confocal optics elements 288 such as an aperture positioned in an image plane for reducing detection of out-of-focus light, and (3) a reference monitor 290 positioned to detect a portion of the excitation light for correcting for variations (e.g., fluctuations and/or inhomogeneities) in the excitation light.

The light source generally comprises any mechanism for producing light suitable for use in an optical assay, such as a photoluminescence, scattering, and/or absorbance

assay, among others. Suitable light sources include lasers, arc lamps, incandescent lamps, fluorescent lamps, electroluminescent devices, laser diodes, and light-emitting diodes (LEDs), among others. The light source may be capable of use in one or more illumination modes, including continuous and time-varying modes, among others.

The detector generally comprises any mechanism for detecting light transmitted from a sample in an optical assay. Suitable detectors include charge-coupled devices (CCDs), intensified charge-coupled devices (ICCDs), videcon tubes, photomultiplier tubes (PMTs), photodiodes, and avalanche photodiodes, among others. The detector may be capable of use in one or more detection modes, including (a) imaging and point-reading modes, (b) discrete (e.g., photon-counting) and analog (e.g., current-integration) modes, and (c) steady-state and time-resolved modes, among others.

The optical relay structure generally comprises any mechanism for transmitting light between the light source, sample, and detector (or simply the sample and detector in a chemiluminescence assay). Suitable optical relay structures may include mirrors, lenses, and/or fiber optics, among others. Here, the optical relay structure includes a beamsplitter that generally transmits excitation light toward the sample and generally reflects emission light toward the detector.

Figure 9 shows an exemplary embodiment of a combined electrical/optical measurement system, including components as described above. Here, a parallel read-out system is used for confocal-optical recordings, for example, using the chip substrate system of Figure 8. In either order, the chip substrate is placed in an appropriate light beam that is able to excite fluorescent probes of interest, and the sample membranes or

cells are positioned at the individual apertures of the chip. The samples are excited using one parallel light beam, for example, using a 45-degree mirrored beamsplitter. The fluorescent light coming from the biological sample or any associated fluorescent probes is transmitted to an optional filter and confocal optics element to increase the signal-to-noise ratio before being projected onto the light sensitive chip of a CCD camera. The confocal optics element reduces or eliminates out-of-focus light not originating from the sample. The spatial resolution of the CCD chip allows detection of fluorescence from all apertures (and consequently all biological samples) simultaneously, if desired.

More generally, the system may be configured to allow top and/or bottom illumination and/or detection of the sample(s), permitting the following combinations: (1) top illumination and top detection, or (2) top illumination and bottom detection, or (3) bottom illumination and top detection, or (4) bottom illumination and bottom detection. Same-side illumination and detection, (1) and (4), is referred to as "epi" and is preferred for photoluminescence and scattering assays. Opposite-side illumination and detection, (2) and (3), is referred to as "trans" and is preferred for absorbance assays.

Alternatively, or in addition, the system may be configured to allow illumination and/or detection at oblique angles. For example, illumination light may impinge on the bottom of a sample holder at an acute angle (e.g., about 45 degrees) relative to detection. In comparison with a straight-on epi system (light source and detector directed at about 90 degrees to sample holder) or a straight-through trans system (light source directed through sample holder directly at detector), an oblique system may reduce the amount of excitation light reaching the detector.

Suitable systems, and components thereof, for top/bottom and/or oblique illumination are described in the following materials, which are incorporated herein by reference: U.S. Patent No. 5,355,215, issued October 11, 1994; U.S. Patent No. 6,097,025, issued August 1, 2000; and U.S. Provisional Patent Application Serial No. 60/267,639, filed August February 10, 2001.

Example 8: Producing, Sizing, and Binding of Vesicles

This example describes exemplary methods for producing, sizing, and binding lipid vesicles.

A mixture of 100 µL asolectin (Fluka) or egg lecithin (EPC), 50 µL palmitoyloleylphosphatidylglycerol (POPG), and 3 µL dipalmitoyl phosphatidyl-ethanolamine-rhodamine (DPPE-rhodamine) (Molecular Probes, USA) (all 10 mg/mL in chloroform, Avanti Polar Lipids), and 70 µL methanol is dried in a rotary vaporizer (Büchi Rotavapor R-114) at low (400 mm Hg) pressure in a 10 mL round flask to form a film. After a 1-hour incubation under vacuum, to the flask is added either 10 mL H_2O or 10 mL of a buffer solution having a concentration of less than 150 mM of KCl and/or less than 600 mM of sucrose or preferably of sorbitol. After a subsequent 16-hour incubation at 37 °C, the resulting lipid vesicles appear as an almost transparent cloud. The lipid vesicles are aspirated and removed using a 1 mL pipette and stored at 4 °C until further use. Storage of the vesicle solution may be improved by the addition of sodium azide (NaN_3) to a concentration of 0.2% by weight. This vesicle preparation procedure yields mostly (> 90%) unilamellar vesicles, with sizes up to 250 µm. However, some of the

vesicles may contain additional smaller vesicles, which are not relevant for membrane formation.

The ability of the resulting vesicles to establish electrically tight seals against a surface aperture is enhanced by purification of the initial vesicle mixture to remove vesicles and lipid impurities that are smaller than about 10 μm in size. Without such purification, the binding of such smaller vesicles in the vicinity of the aperture may prevent electrically tight sealing of the aperture by large (e.g., larger than 10 μm) vesicles. The vesicles may be sized by dialysis, for example, using a nylon mesh with a 20-μm pore size for at least about 20 hours. If necessary, the membrane fluidity of the resulting vesicles may be lowered, for example, by adjusting the lipid composition so that it includes a higher fraction of low-fluidity lipids and/or by lowering the temperature so that it is closer to the phase-transition temperature of the vesicles (e.g., less than or equal to about 4 °C, or more preferably less than or equal to about 1 °C, for some lipids). The unilamellarity of the resulting vesicle membranes may be demonstrated and/or verified using any suitable analytical technique, such as microscopic analysis using a confocal microscope.[xi]

More generally, vesicles may be produced, sized, and bound using any suitable methods. For example, large unilamellar vesicles (giant unilamellar vesicles, GUVs) may be produced using the hydration method.[xii] Similarly, proteoliposomes may be produced using an appropriately modified hydration method.[xiii] Additional vesicles may be produced using the methods described in U.S. Patent Application Serial No. _____, filed

September 14, 2001, titled EFFICIENT METHODS FOR THE ANALYSIS OF ION CHANNEL PROTEINS, and naming Christian Schmidt as inventor.

Example 9: Electrophoretic Positioning of Vesicles

This example, illustrated in Figure 10, describes exemplary methods for electrophoretically positioning samples such as cells and lipid vesicles. The positioning attainable using these methods may exceed that attainable using gravity sedimentation, in at least the following ways: (1) a decrease in the necessary number of vesicles or cells, (2) an increase in the total rate of membrane formation, and (3) an increase in the probability of a successful membrane setup or cell binding. Vesicles and cells also may be prepositioned prior to electrophoretic positioning to improve performance, for example, by introducing the vesicles or cells to the sample so that they are initially positioned above the aperture.

The electrophoretic positioning methods make use of inhomogeneities in the electric potential and associated electric field surrounding the aperture. Figure 10 shows results of a finite element method (FEM) simulation of the electric potential distribution around a substrate in accordance with aspects of the invention. The substrate includes a 4-μm aperture positioned between parallel electrodes. The field distribution is shown as a series of equipotential lines (corresponding a cross-section through the three-dimensional equipotential surfaces), with a spacing of 4 mV, where the potential difference between the electrodes is 80 mV. The field-line curve is distorted in this simulation from its normal circular form to an elliptical form to reflect leak currents in the edge region of the

carrier chips. The following parameters were used in the simulation: C_{buffer} = 10 mM KCl, V = 80 mV, $d_{aperture}$ = 4 μm, and spacing between the aperture and each electrode = 1 mm.

The electric field associated with an electric potential is minus the spatial rate of change of the potential, i.e., $\mathbf{E} = -\nabla V$. Thus, the electric field is perpendicular to the equipotential surfaces at all positions, pointing in the direction of decreasing potential. Moreover, the electric field is stronger where the equipotential surfaces are closer together, and weaker where these surfaces are farther apart. Consequently, from Figure 10, the electric field points toward the aperture (on one side of the aperture), with a strength that increases with proximity to the aperture.

These electrophoretic positioning methods generally may be used in any system capable of creating and focusing an electric field through an aperture. In preferred systems, the electrodes used to create the field are positioned relatively close together, for example, within about 5-10 mm, reducing the voltage required to create an acceptable electric field. Specifically, the measurement and reference electrodes are located, one above and one below the substrate, at a distance of about 0.2 to 3 mm, preferably about 0.5–2 mm, and more preferably about 0.5 to 1 mm. The clamp voltage generated by these electrodes is not critical; however, it customarily lies in the range V_c = –300 to –300 mV, preferably lies in the range–60 to –100 mV, and more preferably lies in the range–60 to –80 mV. The associated electrophoretic-driving force directs vesicles and cells, following the electric field, toward the aperture. In particular, because the electric field is strongly inhomogeneous, increasing sharply in magnitude with proximity to the aperture, vesicles and cells move automatically toward the aperture. In particular, the fields are most

effective near the aperture (e.g., within about 200 μm of the aperture), so that samples preferably are brought into this range by prepositioning or reach it convectively. For this purpose, a hole (for example, d < 1 mm) may be located in the measurement electrode with respect to the aperture.

The following subsections describe two alternative methods of electrophoretic positioning.

Variation 1:

The offset voltage V_{offset} between the electrodes may be corrected before each measurement. To do so, 5 μL of buffer solution is added directly to the aperture, and the measurement electrode is brought to within about 1 mm from the substrate surface. After a liquid meniscus forms between the surface of the substrate and the electrode, the offset voltage and the capacitance of the system are adjusted to compensate.

A 10-μL dispersion of lipid vesicles subsequently is added to the upper side of the measurement electrode, where the vesicles can sediment through the circular opening located in the measurement electrode. Vesicles that move through the measurement electrode opening may be accelerated directly to the aperture opening under the influence of an electric field generated by the applied electrode voltage, V_M = –50 to –80 mV. In doing so, the focusing achieved, measured in the number of vesicles passing through the aperture opening with unmodified surfaces, is a function of the size of the window (that is, the portion of the SiO_2 layer laid open by etching). Smaller SiO_2 windows (e.g., less than about 45 × 45 μm^2) clearly increase vesicle throughput.

Variation 2:

The offset voltage V_{offset} between the electrodes may be corrected before each measurement. To do so, 5 µL of buffer solution is added between the substrate and the measurement electrode, or alternatively between the substrate and the reference electrode, after which the voltage is determined at which the current flow vanishes, satisfying the expression $I(V_{offset}) = 0$.

A 3-µL dispersion of lipid vesicles subsequently is added to the measurement compartment near the aperture, where, in the case of a plane parallel electrode arrangement, the vesicles can sediment through the circular opening located in the measurement electrode. Vesicles that come into the vicinity (e.g., less than about 200 µm) of the aperture experience a very high field intensity (generally but not necessarily between about 100 kV/m and several kV/m) and are accelerated according to the electric field curve directly to the aperture. After the vesicles bind to the substrate and form an electrically tight seal, they are analyzed electrically.

Example 10: Sealing of Vesicles with Unmodified Surfaces

The example, illustrated in Figure 11, describes the sealing of vesicles, as described above, with unmodified surfaces. In particular, Figure 11 shows a plot of current versus time after the docking or binding of vesicles to a 7-µm aperture in the unmodified surface of a suitable substrate. The plot shows that the addition of Ca^{2+} to a final concentration of 4 mM leads quickly to a tight electrical seal between the vesicle membrane and the substrate surface. Specifically, the addition of Ca^{2+} causes a rapid,

significant drop in current, indicating that the membrane has at least substantially blocked ion pathways through the aperture.

Example 11: Binding and Adsorption of Vesicles on Modified SiO_2 Surfaces

The example, illustrated in Figure 12AB, describes the binding and/or other interactions of vesicles as described above with polylysine-modified SiO_2 surfaces.

The binding between these vesicles and surfaces may be strong and rapid, manifesting itself in less than about 0.5 seconds after an appropriate proximity is reached. The probability of successfully positioning a vesicle and subsequently forming an electrically tight membrane seal is strongly dependent on the size of the aperture, the size of the SiO_2 window, and the number, size, and size distribution of the vesicles in the vesicle solution. If substrates having aperture diameters less than about 2 μm and window sizes greater than about 40 μm are used in conjunction with suspensions of vesicles having a vesicle diameter greater than about 40 μm, the probability of binding and forming an electrically tight seal may exceed 90% ($n > 15$, where n is the number of trials). In general, a decrease in the width of the aperture and an increase in the purity of the vesicle suspensions lead to greater reproducibility in the formation of tight aperture seals, both substrate to substrate and vesicle preparation to vesicle preparation.

Vesicles that bind to the surface subsequently may be drawn out to form substantially flat, defect-free membranes. Fluorescence microscopy studies performed using vesicles labeled with both rhodamine (to label vesicle membranes) and carboxyfluorescein (to label vesicle interiors) show that vesicles flatten and may burst upon binding, forming unilamellar structures, since bound vesicles appear flat and red,

suggesting that carboxyfluorescein has been released. These studies were conducted using polylysine-coated glass and a confocal microscope (LSM 510, Zeiss Jena, Germany). Electrical studies of membrane resistance, R_M, demonstrate that the bound vesicles may form substantially defect-free lipid membranes, since very high membrane resistances (e.g., $R_M > 6.4$ GΩ ($n = 26$)) are measured on the substrates in symmetric 85 mM KCl. An analogous series of measurements in symmetric 10 mM KCl demonstrates binding of the vesicles, after appropriate proximity, in less than about 0.2 seconds, with a probability greater than about 70% ($n > 15$) and a membrane resistance greater than about 10 GΩ.

To promote strong adhesion of the vesicles, the surface of the substrate, in given cases, is coated with an adhesion promoter, for example, polycations.[xiv] For physisorption, for example, an aqueous solution of polycations (e.g., 0.1% poly-l-lysine bromide, MW 100,000, Sigma) may be added to the substrate for about 2-5 minutes directly before the measurement and subsequently rinsed off with measurement buffer solution. The covalent binding of peptide polycations preferably is done using previously activated hydroxyl groups of the quartz surface, for example by means of tosyl chloride (triphenylchloromethane).[xv] Through the modification of the substrate surface, an attraction of vesicles with negative surface charge is achieved, which is completely sufficient for electrically tight seals between the membrane and the substrate surface. Alternatively, the surface also may be modified by other compounds having cation characteristics in the desired pH range, such as, for example, 4-aminobutyl-dimethyl-

methoxysilane. Finally, treating the substrates in O_2 plasma for several minutes before surface modification leads to more consistent surface characteristics.

Figure 12AB shows the time course of vesicle binding and the subsequent development of membranes with very high electrical insulation resistance for a 4-μm aperture (Figure 12A) and a 7-μm aperture (Figure 12B) in a poly-L-lysine-coated SiO_2 substrate. The measurements were performed in the presence of 10 μM KCl, using a clamp voltage of –80 mV.

Example 12: Membrane Electrical Properties

This example describes electrical factors relating to preferred aperture sizes, including characteristic electrical properties.

The thermal noise σ of a circular lipid membrane is proportional to $R_M^{-1/2}$:[xvi]

$$\sigma = \sqrt{\frac{4kTf_c}{R_M}}$$

where $R_M = R_{spec}/(\pi r_M^2)$. It follows therefrom that

$$\sigma = r_M \sqrt{\frac{4\pi kTf_c}{R_{spec}}}$$

In these formulae, σ is the effective noise flow, r is the radius, f is the frequency, k is the Boltzmann constant, R is the resistance, and T is the temperature.

Thus, to be a usable membrane for measurement purposes, $r_M/\sqrt{R_{spec}}$ should be very small. The minimization of this product can be pursued according to the invention in two ways: (1) by minimizing the membrane radius r_M, and/or (2) by the electrically tight sealing of the membranes used.

Example 13: Electrical Parameters of Lipid Membranes

This example describes the effects of vesicle fusion on electrical parameters of the measurement system, as described above.

The resistance across the sample substrate changed significantly following vesicle fusion. Before fusion, the resistance is up to 1 MΩ (usually < 450 kΩ) in 85 mM KCl, and similarly usually up to 1 MΩ in 1 mM KCl, depending in both cases on the size of the aperture. Greater resistances are interpreted as artifacts, possibly reflecting, for example, the inclusion of air bubbles under the aperture opening. After fusion, the resistance is greater than about 6.4 GΩ in 85 mM KCl, greater than about 10 GΩ in 10 mM KCl, and greater than about 40 GΩ in 1 mM KCl, corresponding to four order-of-magnitude increases in resistance. Here, the resistance R is at least approximately related to the applied voltage V and the current I according to Ohm's law, i.e., R = V/I.

The capacitance of the sample substrate changed only insignificantly following fusion, by several pF in 85 mM KCl, and by 160 to 280 pF in 1-10 mM KCl.

Example 14: Vesicle Passage through Micrometer Pores

This example, illustrated in Figure 13, describes the passage of vesicles through apertures in the substrate.

The passage of vesicles through an aperture in the presence of negatively charged surfaces, such as unmodified SiO_2 layers, can be observed by monitoring changes in current (or, equivalently, resistance) across the aperture. Specifically, the passage of vesicles will lead to a decrease in current and an associated increase in resistance. To

monitor for artifacts in the observed values, the polarity of the voltage is reversed, whereupon no modulation of current or resistance is observed.

The duration of observed changes in the amplitude of resistance can be correlated with size of the vesicles passing through the aperture being monitored. For example, modulations in the amplitude of resistance lasting up to 18 seconds suggest the passage of very large vesicles with sufficiently fluid membranes. Where vesicle populations with diameters greater than about 50 μm (n = 4) are used in conjunction with aperture openings with diameters of about 7 μm, an almost exclusive variation of the time of passage for fixed changes in amplitude as a function of vesicle size is observed. It is presumed that vesicles undergoing passage through the aperture opening are drawn out during their passage to form tubular structures with definite diameters and closed surfaces.

By analyzing the typical time of passage for large vesicles ($d_{vesicle} >> d_{aperture}$) and the typical change in the amplitude of resistance for small vesicles ($d_{vesicle} \sim d_{aperture}$), the composition of the vesicles with respect to size can be determined for a given vesicle solution. The method of the invention therefore possesses utility for analyzing size distributions in selected populations of vesicles and cells.

Example 15: Observation of Alamethicin Pores and Nicotinic Acetylcholine Receptors

This example, illustrated in Figures 14 and 15AB, describes observation of electrical activity of alamethicin pores and nicotinic acetylcholine receptors (nAChR) in fused vesicles. These observations confirm the biological utility of the invention.

Figure 14 shows a plot of current versus time for membranes containing alamethicin. In these experiments, a membrane is formed over an aperture in 85 mM KCl. Next, alamethicin is added to the measurement compartment, to a final concentration of 0.1 μg/mL.[xvii] Finally, a potential is applied, and a plot of current versus time is generated. The amplitude and dwell times of the current fluctuations observed in this plot, corresponding to 600 pS conductivities of the alamethicin pores, prove the functionality and high sensitivity of the system.

Figure 15AB shows an analogous plot of current versus time for membranes containing the membrane protein nAChR. In these experiments, a membrane is formed as above. Next, nAChR is introduced into the membrane via Ca^{2+}-mediated fusion. Specifically, nAChR is purified from an appropriate source, and then reconstituted into small unilamellar vesicles.[xviii] Next, these vesicles are added to the measurement compartment, and then fused to the membrane by increasing the Ca^{2+} concentration of the sample chamber to greater than about 1 mM. In given cases, the fusion is supported by the subsequent temporary setup of an osmotic gradient.[xix] Finally, a potential is applied, and a plot of current versus time is generated. In the absence of agonists, typical receptor opening events are observed (Figure 15A), whereas, in the presence of agonists, such as carbamylcholine (20 μM final concentration), such receptor opening events are substantially extinguished within a short time ($t < 100$ seconds) (Figure 15B).

Example 16: Analysis of Cells

This example, illustrated in Figures 16-18AB, describes use of the above-described systems in the study of cells.

The methods of the invention are generally applicable to the investigation and analysis of cells. Such cells may be positioned and electrically characterized using procedures substantially analogous to those described above for vesicles. However, in some cases, the positioning and measurement methods may be modified as desired to accommodate differences in vesicles and cells, including, among others, the cytoskeleton, the varied lipid and protein content of cell membranes, and the cell wall in plants and certain algae, bacteria, and fungi. For example, the cell may be made more flexible by disrupting the cytoskeleton, for example, using cytochalasin and/or colchicine. Similarly, in measurements using plant cells, the cell wall may be removed to expose a relatively smooth membrane surface capable of forming a tighter electrical seal. Similarly, in measurements using animal cells derived from tissues, the extracellular matrix may be removed or digested, for example, using one or more proteases, lipases, and/or glycosidases, among others.

The methods may be used for a variety of patch clamp experiments, in a variety of formats or configurations. The cell, as initially bound and sealed, is in a "cell-attached configuration." If the membrane patch over the aperture then is ruptured or destroyed, for example, by applying a pulse of voltage or suction, electrical measurements can be performed over the entire cell membrane in a "whole-cell configuration." Alternatively, if the membrane patch over the aperture is permeabilized, for example, by the addition of pore formers such as amphotericin B or nystatin to the reference compartment, electrical measurements again may be performed over the entire cell membrane in a "perforated-patch configuration." Alternatively, if the cell (instead of the membrane patch over the

aperture) is lysed, electrical properties of the patch may be measured in an "inside-out configuration." In the lattermost approach, the cytosolic side of the membrane is exposed to the measurement solution, and the relatively small area of membrane being analyzed potentially makes possible the study of individual channel events.

Figures 16-18 show exemplary results of positioning and voltage clamp experiments performed using Jurkat cells. These cells, a human mature leukemic cell line, phenotypically resemble resting human T lymphocytes and are widely used to study T cell physiology. Similar results (not shown) were obtained using TE 671 cells and CHO cells.

The cells were cultured and prepared using standard cell culture techniques. These cells were maintained for 2-5 days at 37°C in 5% CO2 in RPMI with Glutamax, supplemented with 10% FCS and P/S (100U/100µg / mL). Before use, cells were resuspended in a physiological buffer (PB = NaCl, 140 mM; HEPES, 10 mM; KCl, 5mM; $CaCl_2$, 2 mM; $MgCl_2$, 1.2 mM; pH 7.3, osmolarity 290 mOsm) at a density of 10^7 cells/mL. Lower and upper fluid compartments were filled with 20 µL and 15 µL of PB, respectively. Five µL of the cell suspension was added to the upper compartment. Positioning was made at V_m= -60 or -90 mV. All experiments were performed at room temperature.

Figure 16 shows the time course of positioning, binding, and subsequent development of a tight electrical seal for a Jurkat cell. The cell was positioned at -60 mV. Seal formation occurred about 15 seconds after cell addition, quickly rising to about 1000 GΩ. The aperture size was about 3 µm, and the chip resistance was about 250 kΩ.

Figure 17 shows a series of plots of current versus time showing the current flowing through the membrane of a Jurkat cell at the indicated positive and negative clamp voltages in a cell-attached configuration. The curves appear quantized, switching largely between just two values, one low and one high, particularly at higher voltages. This characteristic suggests that single-channel events are being observed, corresponding to the opening and closing of the channel.

Figure 18 shows an analysis of the current flowing through the membrane of a Jurkat cell for a +60 mV clamp voltage. Panel A shows a representative plot of current versus time. This plot again has a quantized character, like Figure 17. Panel B shows a histogram plotting the relative occurrence of a given current versus the current. The histogram is bimodal, with peak values of about 1.3 pA (the relatively smaller peak at left) and about 4.8 pA (the relatively larger peak at right), corresponding to an average of about 3.5 pA.

Example 17: Miscellaneous Applications

The positioning and measurement systems provided by the invention may be used for a variety of purposes and a variety of assays. Exemplary miscellaneous applications are described below.

Screening of Ingredients

The system may be used to screen libraries according to any suitable criterion, such as the identification of candidate drugs, modulators, and the like. Suitable libraries include compound libraries, combinatorial chemistry libraries, gene libraries, phage libraries, and the like. The system is exceptionally well suited to probing libraries whose

members are present only in small amounts, such as (1) the large number of potential ligands that can be produced using combinatorial chemistry, and (2) many receptor proteins, above all ligand-controlled and G-protein-coupled receptors (GPCRs). Owing to the process according to the invention, or alternatively the measurement arrangement/measurement apparatus according to the invention, it is possible to work with very few cells, either directly or after previous isolation and reconstitution of the receptor proteins in vesicles or lipid membranes. By the uncomplicated arrangements of the sensor elements in arrays, different substances or receptors can be selected simultaneously. There is moreover the possibility of receptor cleaning and reconstitution in lipid vesicles microchromatographically in on-chip containers that optionally may be integrated into the apparatus according to the invention.

Replacement of Conventional Patch-clamp Technologies

Conventional patch-clamp technologies form the foundation of the investigation of the functionality of membrane receptors as well as the modification of membrane characteristics as a response to signal and metabolic processes in cells. If isolated cells of a homogeneous cell population serve as the object of the investigation, as is, for example, often the case in transformed cells, the process according to the invention serves as an at least comparable replacement for the patch-clamp technologies. As objects of investigation for this process, for example, dissociated neurons and cultivated mammalian cells as well as plant protoplasts are suitable.

Portable Biosensors/Environmental Analytics

The automation and outstanding mechanical stability of the measurement system according to the invention permits its use in biosensors. By using suitable transformed cells, receptors reconstituted in vesicles, or channel-forming proteins, sensors can be set up that are sensitive to very different substrates or metabolites. Moreover, if sufficiently tight electric seals are formed, which is possible using the apparatus according to the invention, then measurement sensitivity will in principle only be dependent on the binding constant of the receptor. This sensitivity may lie under one nanomole for G-protein-coupled receptors, and in the nanomolar range for ionotropic receptors (e.g., 5 HT3, nAChR, $GABA_AR$, glycine R, and GluR).[xx]

The disclosure set forth above may encompass multiple distinct inventions with independent utility. Although each of these inventions has been disclosed in its preferred form(s), the specific embodiments thereof as disclosed and illustrated herein are not to be considered in a limiting sense, because numerous variations are possible. The subject matter of the inventions includes all novel and nonobvious combinations and subcombinations of the various elements, features, functions, and/or properties disclosed herein. The following claims particularly point out certain combinations and subcombinations regarded as novel and nonobvious. Inventions embodied in other combinations and subcombinations of features, functions, elements, and/or properties may be claimed in applications claiming priority from this or a related application. Such claims, whether directed to a different invention or to the same invention, and whether

broader, narrower, equal, or different in scope to the original claims, also are regarded as included within the subject matter of the inventions of the present disclosure.

[i] (J. P. Changeux (1993), "Chemical Signaling in the Brain," Sci. Am. Nov. Pages 30 ff; A. G. Gilman (1995), Angew. Chem. Int. Ed. Engl. 34: 1406–1428; M. Rodbell (1995), Angew. Chem. Int. Ed. Engl. 34: 1420–1428)

[ii] (J. Hodgson (1992) Bio/Technology 9: 973)

[iii] (J. Knowles (1997) "Medicines for the New Millennium Hunting Down Deseases" [sic: Diseases] Odyssey Vol. 3 (1))

[iv] (O. P. Hamill, A. Marty, et al. (1981), "Improved Patch-clamp Techniques for High-resolution Current Recording from Cells and Cell-free Membrane Patches" Pflugers Arch 391(2): 85–100)

[v] (Radler, J., H. Strey, et al. (1995), "Phenomenology and Kinetics of Lipid Bilayer Spreading on Hydrophilic Surfaces." Langmuir 11(11): 4539–4548)

[vi] For example, polycations, such as described by Mazia, Schatten, et al. (see D. Mazia, G. Schatten et al. (1975), "Adhesion of Cells to Surfaces Coated with Polylysine." J. Cell Biol. 66: 198 to 200).

[vii] (J. Edelstein, O. Schaad, J.-P. Changeux (1997), "Single Binding versus Single Channel Recordings: A New Approach to Study Ionotropic Receptors," Biochemistry 36: 13755–13760)

[viii] (see Hamill, Marty, et al. 1981 loc. cit; J. G. Nicholls, A. R. Martin, et al. (1992), From Neuron to Brain: A Cellular and Molecular Approach to the Function of the Nervous System, Sunderland, Ma., Sinauer Associates, Inc.)

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[x] Z. M. Pei, J. M. Ward, et al. (1996), "A Novel Chloride Channel in Vicia Faba Guard Cell Vacuoles Activated by the Serine/Theonine Kinase, CDPK," EMBO J. **15**(23): 6564–74

[xi] See, e.g., R. B. Gennis (1989), Biomembranes: Molecular Structure and Function, New York, Berlin, Heidelberg, Springer Verlag.

[xii] H. H. Hub, U. Zimmerman, et al. (1982), "Preparation of Large Unilamellar Vesicles," FEBS Lett. **140**(2): 254–256; P. Mueller, T. F. Chien, et al.(1983). "Formation and Properties of Cell-like Lipid Bilayer Vesicles," Biophys. J. **44**(3): 375–81, K. Akashi, H. Miyata, et al. (1996), "Preparation of Giant Liposomes in Physiological Conditions and their Characterization under an Optical Microscope," Biophys. J. **71**(6): 3242–50

[xiii] M. Criado and B. U. Keller (1987), "A Membrane Fusion Strategy for Single-channel Recordings of Membranes Usually Non-accessible to Patch-clamp Pipette Electrodes," FEBS Lett. **224**(1): 172–6; B. U. Keller, R. Hedrich, et al. (1988), "Single-channel Recordings of Reconstituted Ion Channel Proteins: An Improved Technique," Pflugers Arch **411**(1): 94–100

[xiv] see Mazia, Schatten, et al. loc. cit., 1975

[xv] M. L. Williamson, D. H. Atha, et al. (1989), "Anti-T2 Monoclonal Antibody Immobilization on Quartz Fibers: Stability and Recognition of T2 Mycotoxin," Analytical Letters 22(4): 803-816

[xvi] B. Sakmann and E. Neher (1983), Single-channel Recording, New York, London, Plenum Press

[xvii] R. B. Gennis (1989), Biomembranes: Molecular Structure and Function, New York, Berlin, Heidelberg, Springer Verlag

[xviii] Schürholz (Schürholz, T., J. Kehne, et al. (1992), "Functional Reconstitution of the Nicotinic Acetylcholine Receptor by CHAPS Dialysis Depends on the Concentration of Salt, Lipid, and Protein," Biochemistry **31**(21): 5067–77; Schürholz, T. (1996), "Critical Dependence of the Solubilization of Lipid Vesicles by the Detergent CHAPS on the Lipid Composition. Functional Reconstitution of the Nicotinic Acetylcholine Receptor into Preformed Vesicles above the Critical Micellization Concentration," Biophys. Chem. **58**(1-2): 87–96).

[xix] See: Eray, Dogan, et al. 1995 loc. cit.

[xx] (North, R. A. (1994), Ligand- and Voltage-gated Ion Channels, CRC Press; Peroutka, S. J. (1991), Serotonin Receptor Subtypes–Basic and Clinical Aspects, New York, John Wiley & Sons; Peroutka, S. J. (1994), G-protein-coupled Receptors, CRC Press; Conley, E. C. (1996), The Ion Channel Facts Book, Academic Press)

Claims

1. Multiaperture biochip for positioning and/or analyzing samples such as cells, vesicles, cellular organelles, and fragments, derivatives and mixtures thereof, said biochip comprising a substrate including several apertures, at least one recording fluid compartment and one reference fluid compartment arranged on each side of said substrate and being in contact via said apertures, at least one recording electrode and one reference electrode in contact with at least one of said compartments and adapted to measure and/or apply an electrical potential across said apertures, characterized by the fact that it furthermore comprises a support element for independently supporting said substrate and associated system components.

2. Multiaperture biochip according to claim 1 wherein said support element includes a carrier plate and at least one spacer sandwiched between the substrate and the carrier plate.

3. Multiaperture biochip according to claim 2 wherein the space defined between said substrate and said carrier plate includes at least one of said compartments.

4. Multiaperture biochip according to any of the previous claims wherein several fluid compartments are arranged on one side of the substrate, each of those compartments being in contact, via said apertures, with one single compartment arranged on the other side of the substrate.

5. Multiaperture biochip according to any of claims 2 to 4 wherein the carrier plate is made of a transparent material, such as glass, and includes a plurality of microlenses arranged in such a way that they allow the parallel optical observation of the samples which are positioned near the apertures.

6. Multiaperture biochip according to any of the previous claims wherein at least one electrode is arranged adjacent to the substrate.

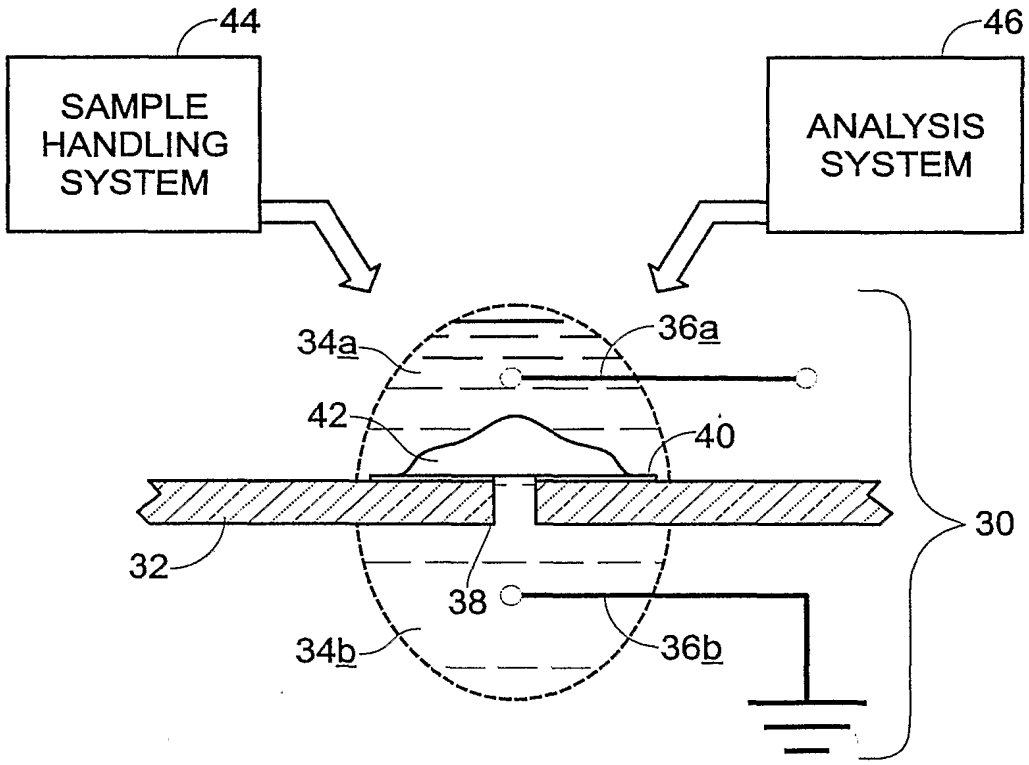
7. Multiaperture biochip according to any of the previous claims wherein all the recording electrodes are contacting the substrate or the support element at its border.

8. Multiaperture biochip according to any of the previous claims wherein said recording fluid compartment is arranged above or below the substrate.

9. Multiaperture biochip according to any of the previous claims wherein it contains 96 or 384 or 1536 apertures.

10. Multiaperture biochip according to any of the previous claims wherein at least one compartment has a dome shape formed by the fluid itself, said domed shape resulting from surface tension.

Fig. 1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ANALYSIS SYSTEM
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36b
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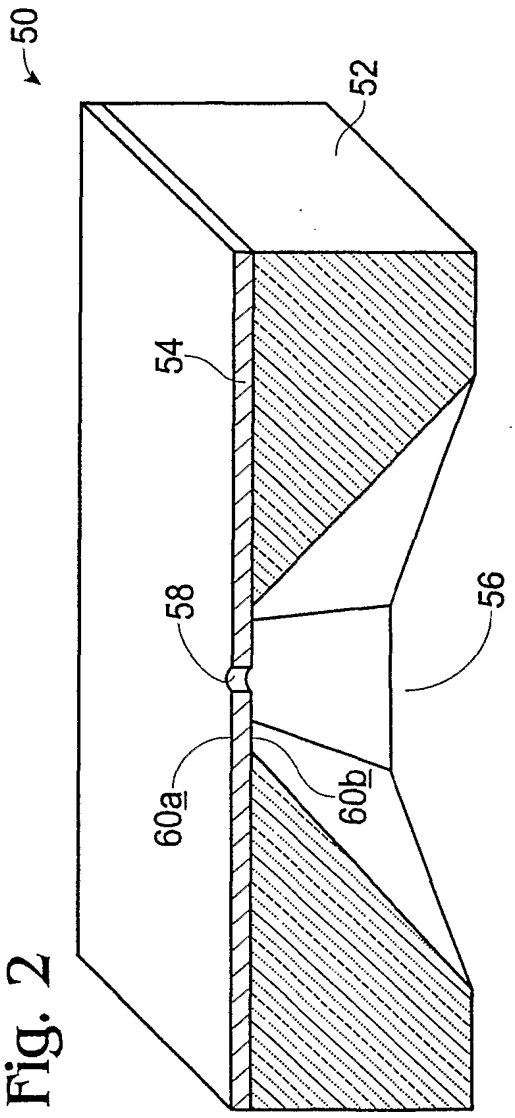
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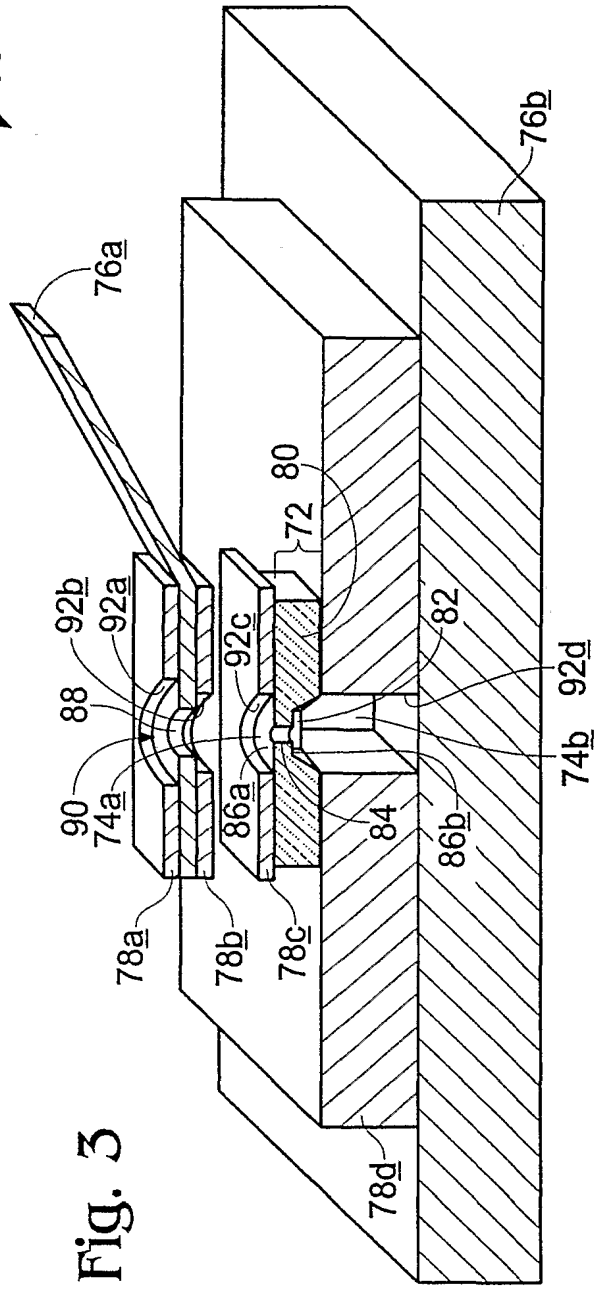
Fig. 2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78d

Fig. 3

Fig. 4

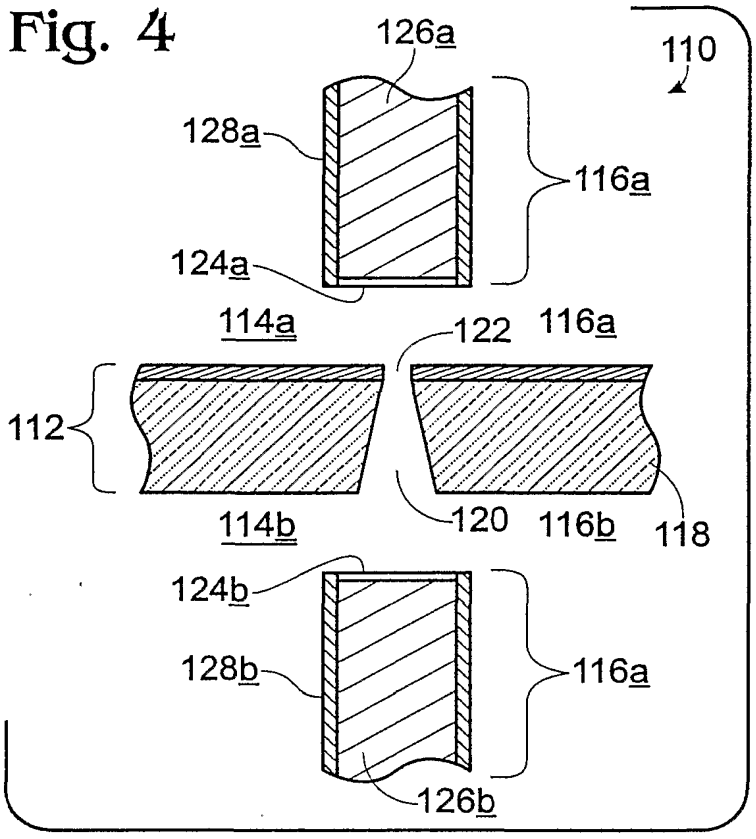
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Fig. 5

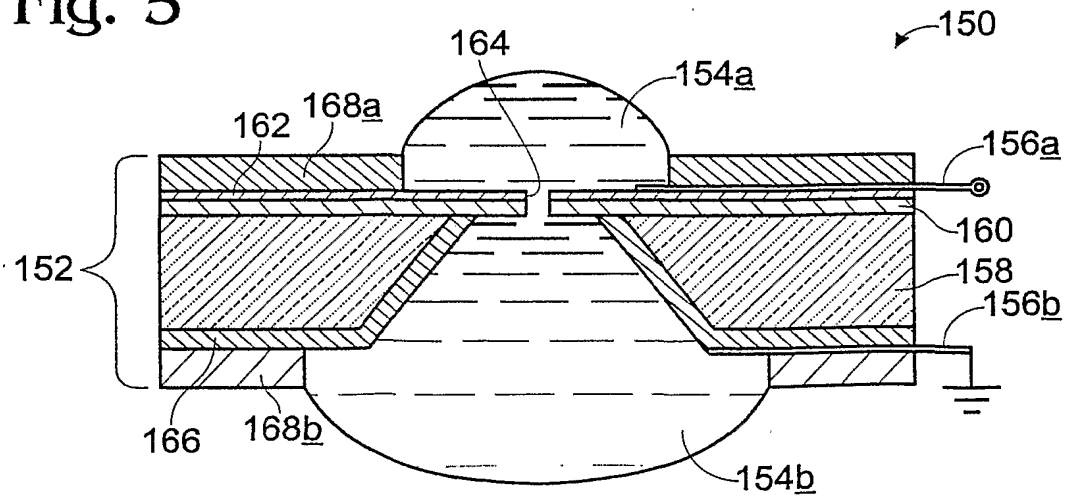
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Fig. 6

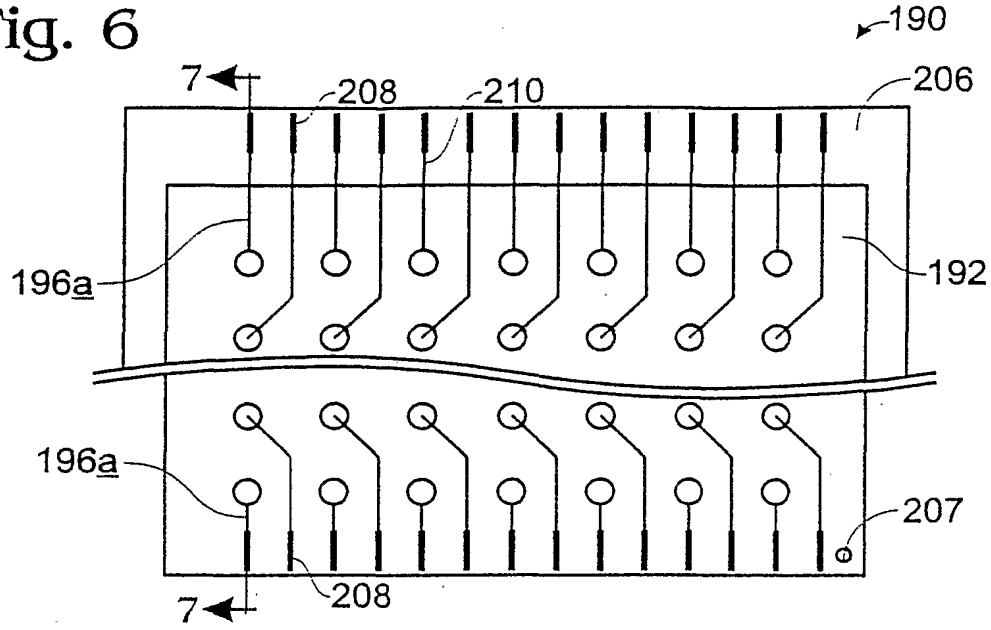


Fig. 7

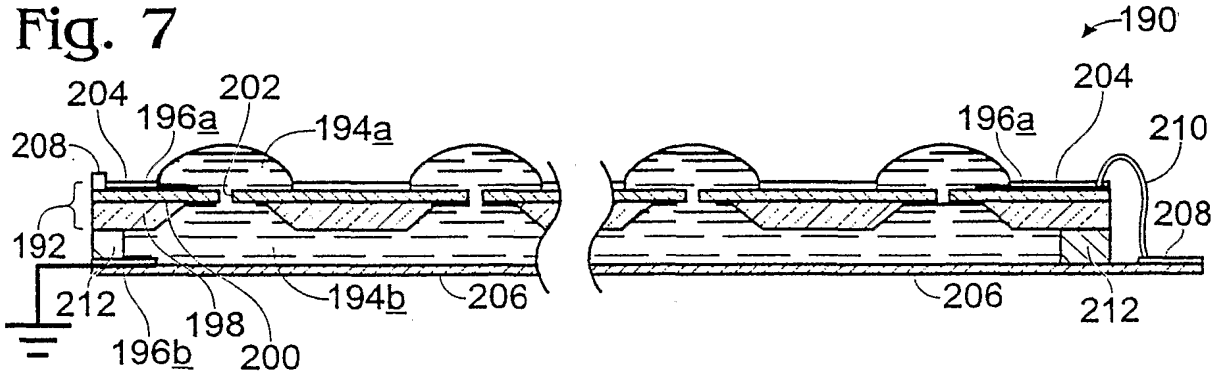


Fig. 8

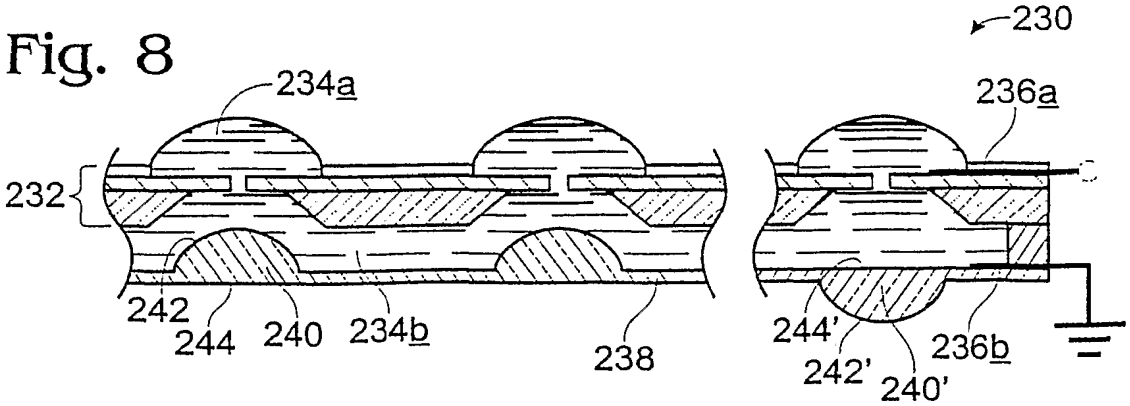


Fig. 9

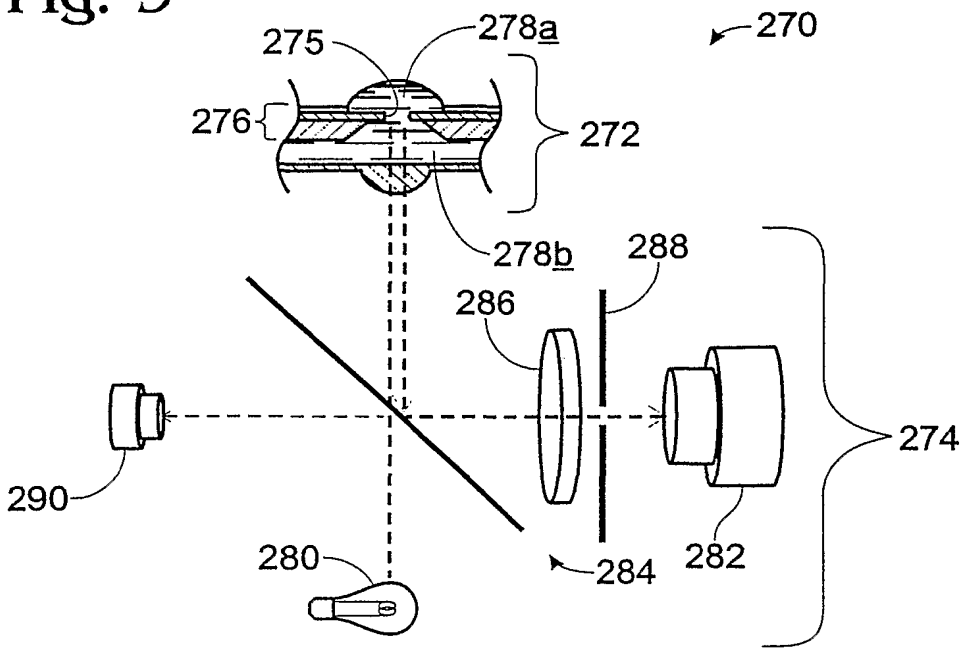


Fig. 10

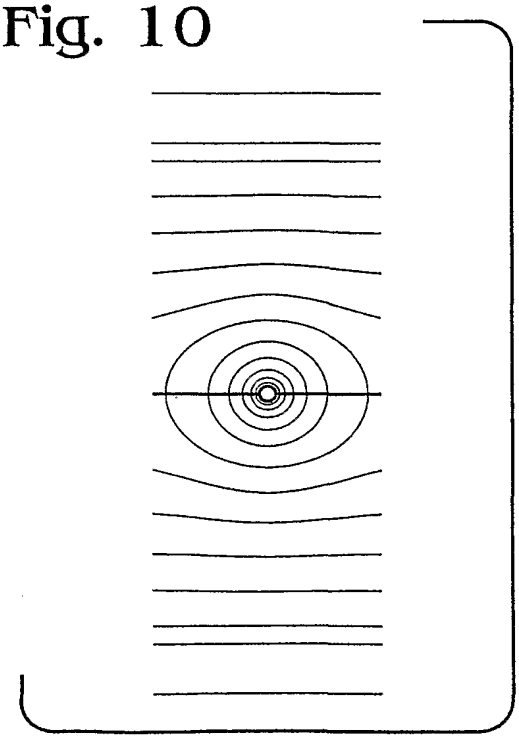


Fig. 11

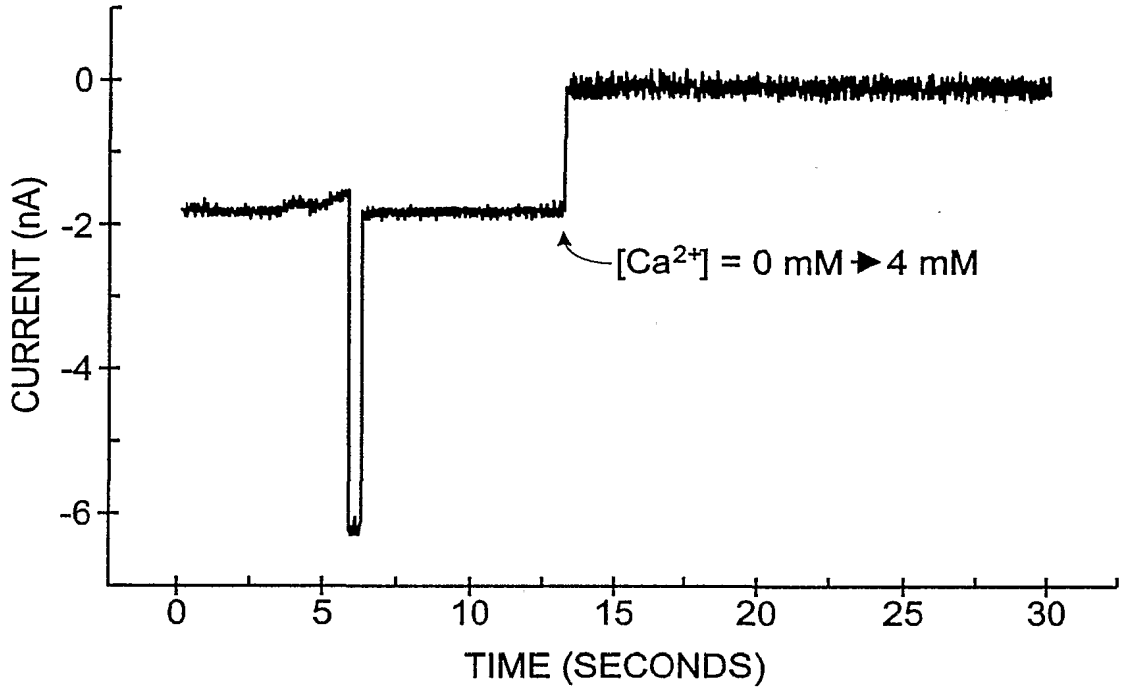


Fig. 12A

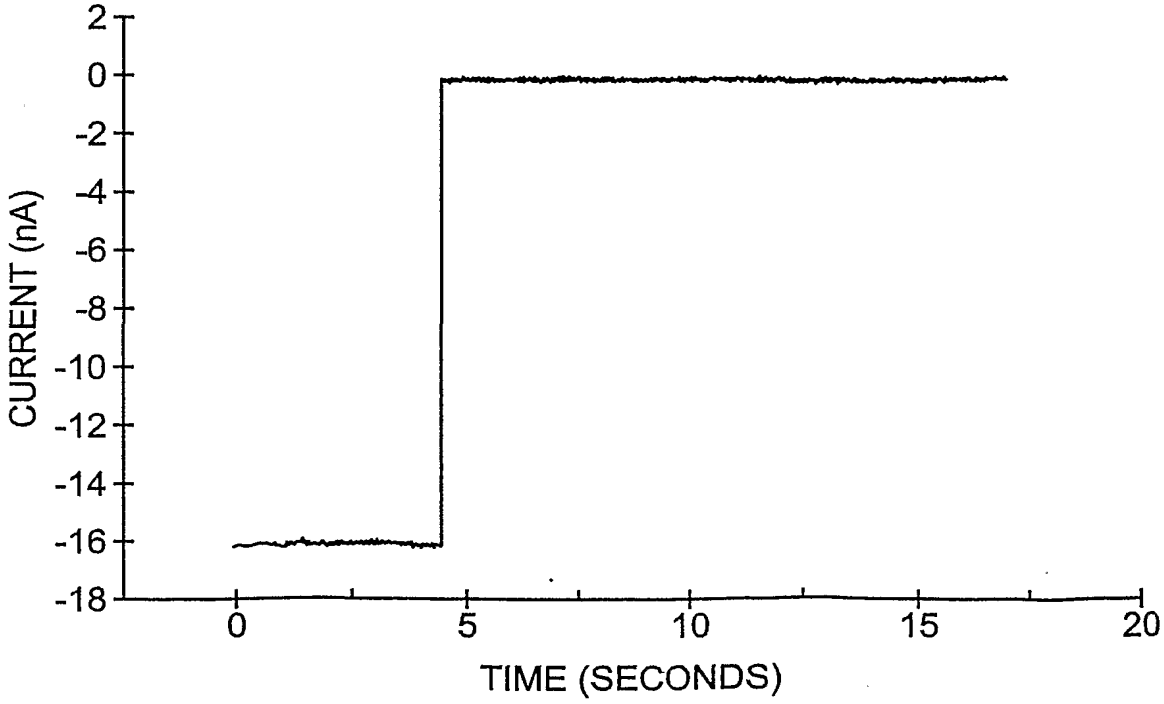


Fig. 12B

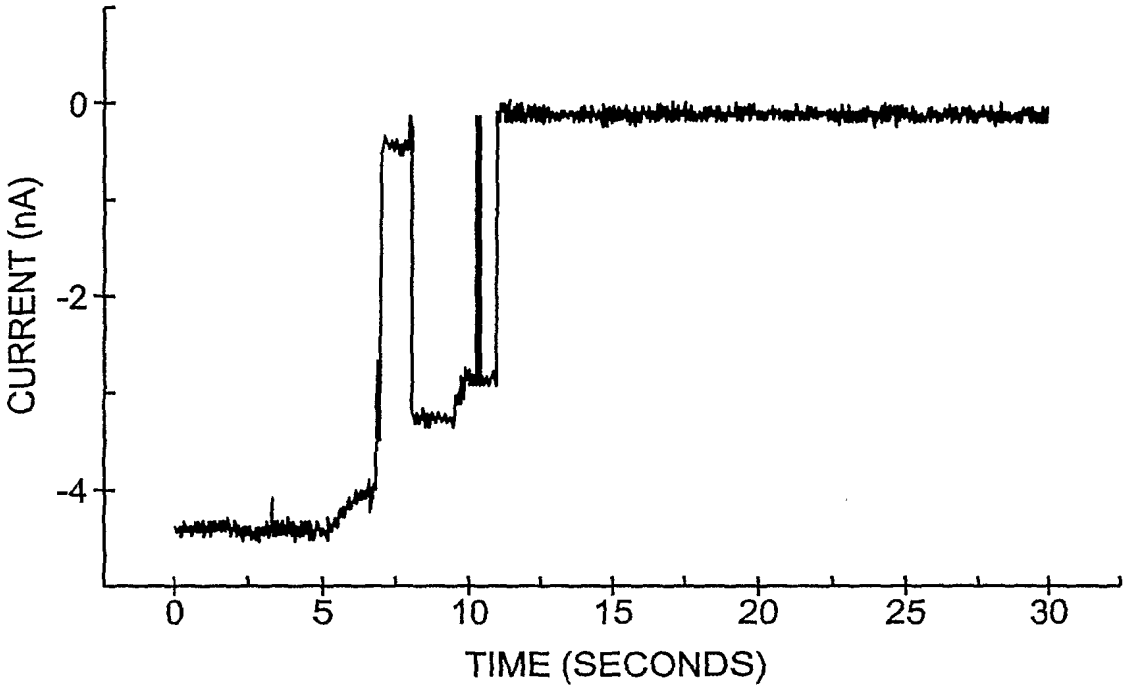


Fig. 13

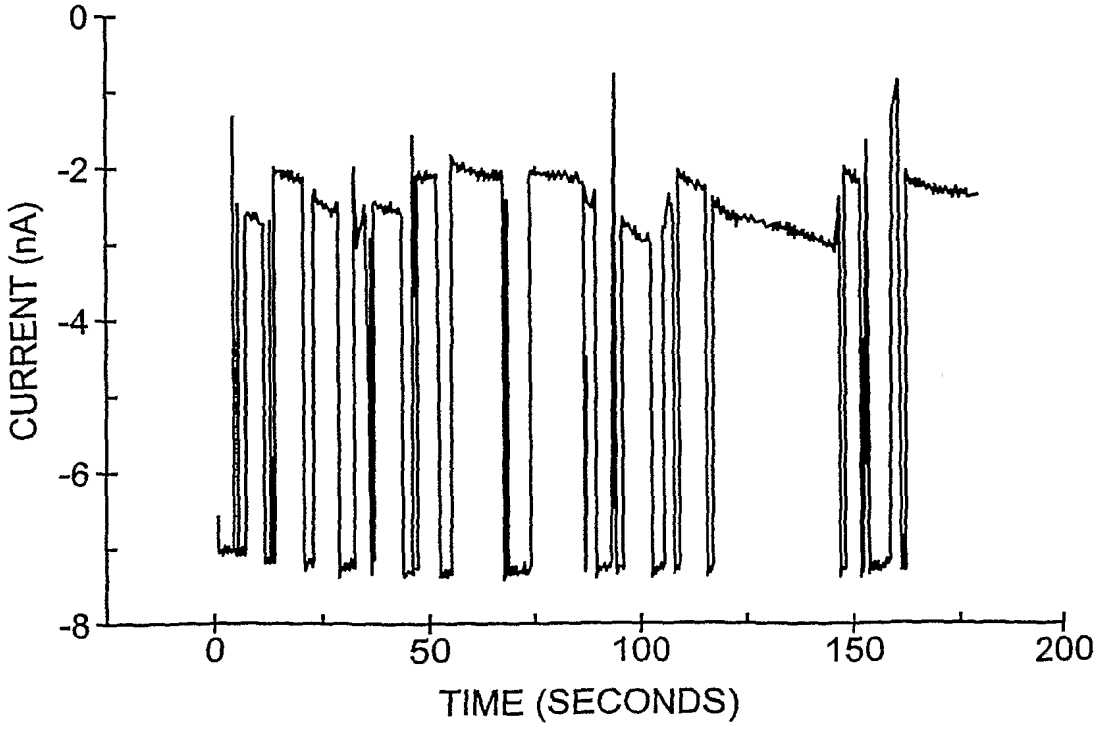


Fig. 14

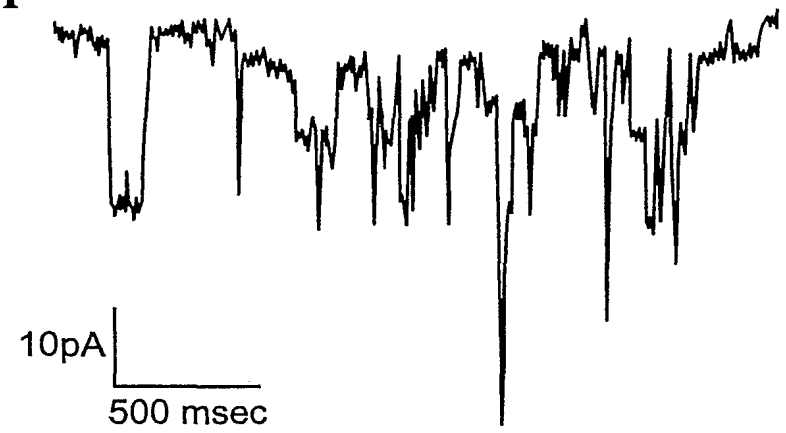


Fig. 15A

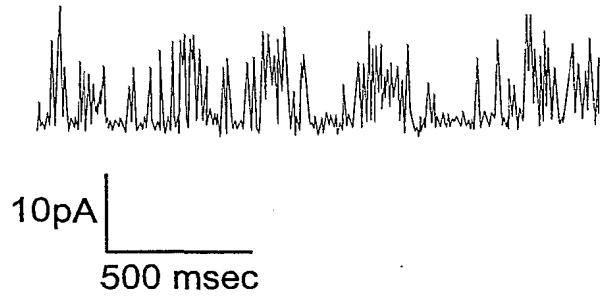


Fig. 15B

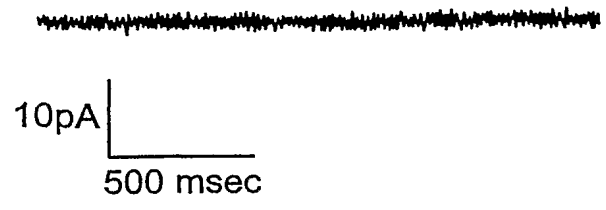
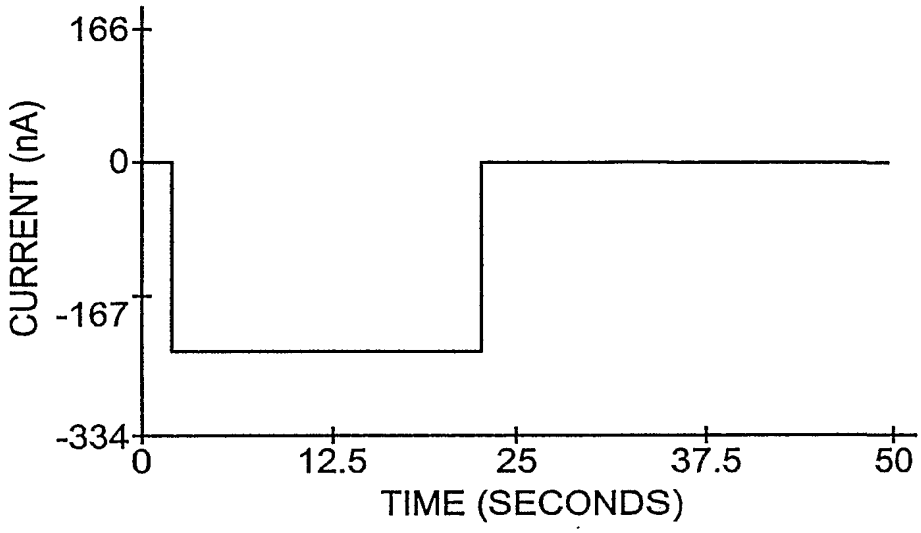


Fig. 16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0
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CURRENT (nA)
0
12.5
25
37.5
50
TIME (SECONDS)

Fig. 17

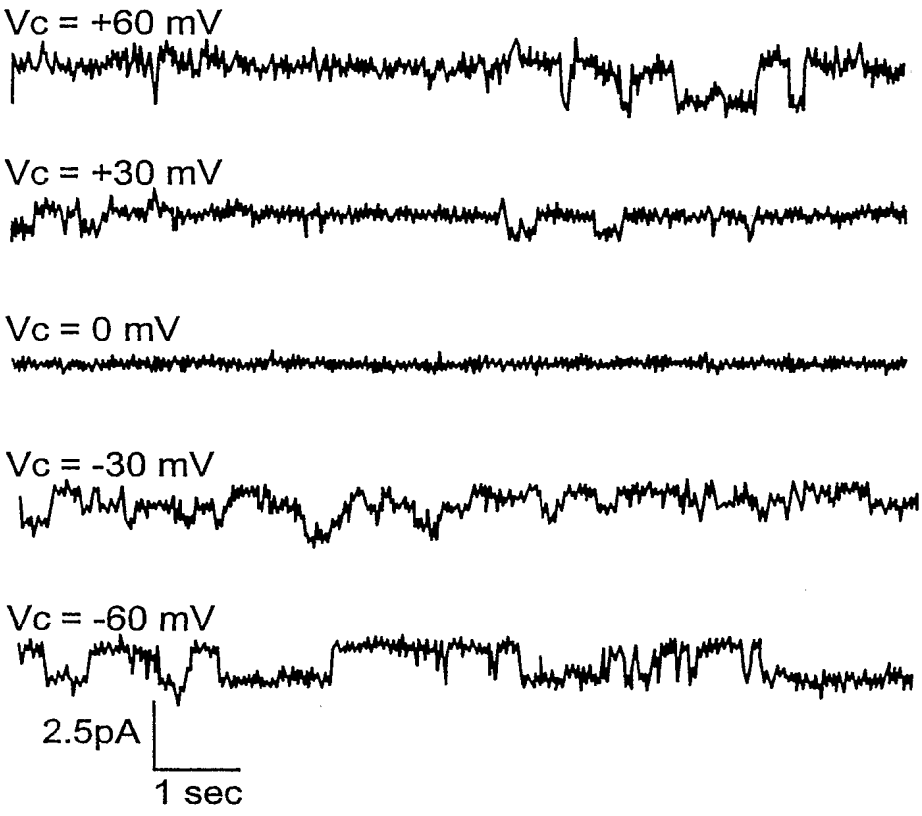
Vc = +60 mV
Vc = +30 mV
Vc = 0 mV
Vc = -30 mV
Vc = -60 mV
2.5pA
1 sec

Fig. 18A

Fig. 18B